Combination of Dasatinib and Dexamethasone targets the myeloma plasma cell through its interaction with the bone marrow microenvironment

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Abbreviations

ABCB1 Adenosine triphosphate (ATP)-binding cassette (ABC) transporter B1

AP-1 Activated protein-1

APRIL A proliferation inducing ligand

BAFF B cell activating factor

bFGF basic fibroblast growth factor

BMP-2 Bone morphogenetic protein 2

CAM Cell adhesion molecules

CAM-DR Cell adhesion mediated drug resistance

CAS CRK-associated substrate

CFSE Carboxy fluorescein succinimidyl ester

CI Combination Index

CML Chronic myeloid leukaemia

Crkl Crk like protein

CSK c-Src tyrosine kinase

DKK1 Dickkopf 1

ECM Extra cellular matrix

EGTA ethylene glycol tetra acetic acid

FAK Focal Adhesion Kinase

FGFR3 Fibroblast growth factor receptor 3

FI fluorescence intensity

FISH Fluorescent in situ hybridisation

GEP Gene expression profiling

HGF Hepatocyte growth factor

HMCL Human myeloma cell lines

HUVEC human umbilical vein endothelial cells

IGF-1 Insulin growth factor -1

LFA1 Lymphocyte function-associated antigen 1

LRP Lung resistance protein

MAGE Melanoma associated antigen

MAPK mitogen-activated protein kinase

MGUS Monoclonal gammopathy of undetermined significance

MIP-1α Macrophage inflammatory protein

MMEC Multiple myeloma endothelial cell

MMP-9 Matrix metalloproteinase -9

MRP1 Multidrug-resistance associated protein

NKT Natural killer T cell

ONJ Osteonecrosis of the jaw

OPG Osteoprotegerin

OS Overall survival

PFA Paraformaldehyde

PFS Progression free survival

PTHrP parathyroid hormone (PTH)-related peptide

PTP1B protein tyrosine phosphatase 1B

RANKL nuclear factor (NF)-κB (RANK) ligand

RGD arginine-glycine-aspartate

RHAMM Receptor for hyaluronan-mediated motility

RNAi systematic RNA interference

SDF-1α Stromal derived factor alpha

SFK Src family protein kinases

sFLC Serum Free light chain

sFRP-2 Secreted Frizzled-related protein 2

SiRNA short interfering RNA

SREs Skeletal related events

STAT3 signal transducer and activator of transcription 3

TTP Time to progression

VEGF Vascular endothelial growth factor

ABSTRACT

Abstract:

Dexamethasone therapy has been the backbone of myeloma treatment for the last few decades. Despite advances in chemotherapy, myeloma continues to be incurable and all patients eventually relapse with progressive disease. Cell adhesion mediated drug resistance and a permissive bone marrow microenvironment facilitates myeloma cell survival. Upto 90% of myeloma patients present with bone disease of varying degrees, which is a result of enhanced osteoclastic activity and reduced osteoblast differentiation and function. Dasatinib, a multi-targeted tyrosine kinase inhibitor, inhibits the Src family kinases, Abl kinase, c- kit and PDGFRβ. I hypothesised, combining Dasatinib with a cytotoxic agent dexamethasone; through direct antimyeloma effect, disruption of osteoclast cytoskeleton and adhesion of plasma cells to the bone marrow microenvironment, would enhance tumour sensitivity to steroids.

In plasma cells, combination of Dasatinib and dexamethasone at clinically relevant concentrations induced apoptosis of human myeloma cell lines and exhibited synergy. Cell cycle analysis showed reduction in the proliferative compartment (G2/M) and increase in the non-viable (SubG0) population indicating cell death by apoptosis also confirmed by Annexin V staining. Dasatinib and Dexamethasone combination inhibited secretion of IL-6 in cocultures of fibroblastic stromal cells and plasma cells but not MIP – 1 α in myeloma cells.

Dasatinib inhibited adhesion of plasma cells on Fibronectin (FN) despite integrin activation. This effect correlated with down regulation of Src and Abl activity. Both Dasatinib and Dexamethasone inhibited adhesion of PC on

stromal cells and osteoclasts. Dasatinib disrupted the organisation of actin cytoskeleton in osteoclasts and blocked osteoclast maturation. Similar effect was observed with PP2 a Src inhibitor, confirming that the effects were mediated at least in part through Src inhibition. This effect resulted in impaired osteoclast function, evidenced by reduced *in vitro* bone resorption. A novel *in vitro* coculture system comprising eGFP expressing MM cell lines was designed and validated with available methods to screen drugs when plasma cells were cocultured with accessory cells in the microenvironment. Using this system, I found the combination of dasatinib with dexamethasone overcomes the protective effect of fibroblastic stromal cells and osteoclasts and induces apoptosis of myeloma cells. I conclude that Dasatinib would overcome resistance of myeloma cells to dexamethasone in the presence of stromal cells and osteoclasts. These findings warrant exploring this drug combination in steroid resistant myeloma patients with extensive skeletal disease in a phase I/II trial.

INTRODUCTION

Introduction

Myeloma is an incurable neoplasm characterised by the accumulation of clonal plasma cells in the bone marrow accounting for approximately 1% of all cancers (10-15% of haematological malignancies). In the year 2000, the annual age-standardised incidence in South East Thames region was 7.8 per 100,000 of the population. With a median age of onset of 73 years, Myeloma is predominantly a disease of the elderly [1]. It was estimated that approximately 11,000 patients currently suffer from multiple myeloma in the UK, with approximately 3,000 new cases and 2,000 deaths from the disease each year. The recent data released by office of national statistics has reported 4040 new patients in the year 2007 with an annual age standardised incidence (European) rate per 100,000 population of 4.8 (4.7 -5.0) [2]. The age standardised mortality rate in the year 2008 for myeloma was 2.8 (2.7-3.0). The 5 yr overall survival (OS) rate of 33.1% (32.1 -35.0) between 2003 and 2007, with a survival improvement of 3.5% over a one year period [3].The survival advantage is at least partly explained by availability of novel agents for treatment of myeloma which have direct cytotoxic effect on the neoplastic plasma cells and effects on the bone marrow microenvironment, which is permissive for the plasma cells to proliferate and survive. Smouldering myeloma is an asymptomatic plasma cell disorder associated with a high risk of progression to symptomatic multiple myeloma or amyloidosis. By definition these patients have more than 10% plasma cells in their bone marrow with no evidence of end organ damage. Disease progression occurs in up to 73% of patients as reported in the largest study to date by Kyle et al. They also found

that the risk of progression was related to the levels of monoclonal protein and number of plasma cells in the bone marrow [4]. Overall risk of progression decreases with time, 10% per year for the first 5 years, approximately 3% per year for the next 5 years, and 1% per year for the last 10 years in a 20 yr follow up period. Monoclonal gammopathy of undetermined significance (MGUS) is a benign pre neoplastic stage with bone marrow plasmacytosis less than 10% with no evidence of end organ damage. The rate of progression from MGUS to myeloma is 1% per year and higher in patients with non IgG paraproteinaemia with a high level of monoclonal protein at presentation and abnormal serum free light chain ratio between kappa and lambda light chains [5,6]. Until recently, most patients were thought to present with denovo myeloma and a small proportion progressed from a MGUS. Recently, Landgren et al studied 71 myeloma patients among 77476 adults enrolled in prospective cancer screening trial [7]. Serum protein electrophoresis, immunofixation, and serum Free light chain (sFLC) assays were performed on stored samples to determine the prevalence of MGUS. All the 71 patients who had developed MM had a preceding MGUS, with 3/4th of patients with a detectable monoclonal protein up to 8 or more years prior to the diagnosis of MM. Fifty percent of patients showed a longitudinal increase in monoclonal protein prior to the diagnosis of myeloma. Weiss and colleagues also studied 30 patients with MM where there was serum available stored by the US Department of Defence Serum Repository. A preceding monoclonal gammopathy was detected in 27/30 patients with serial increase in monoclonal paraprotein and light chains in some of the patients where samples were available. The number of patients classified to have non

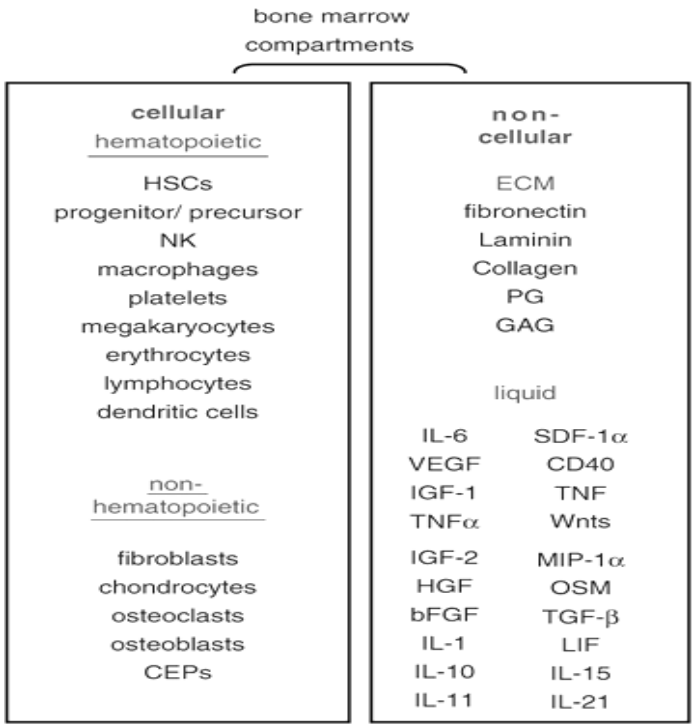
secretory myeloma where a serum or urine paraprotein was not detected has been significantly reduced since the availability of serum free light chain assay. This test has a very high sensitivity and specificity to detect differential monoclonal rise in light chains (normal ratio kappa: lambda – 0.26 - 1.65) as a marker of clonal lymphoproliferative disorder. Dispenzieri and colleagues described a new entity of light chain MGUS where there is absence of a IgH expression[8]. In their study, the prevalence rate of light chain MGUS was 0.8% with a risk of progression to myeloma of 0.3% per 100 person-years. Despite the risk of progression of myeloma the other significant co morbid feature is the development of renal disease in 23% of patients with light chain MGUS [8]. There are no defined therapies for MGUS or smouldering myeloma which would arrest disease progression. On an ethical and practical note such therapeutic trials will be difficult to justify particularly as patients are asymptomatic and the primary end point of disease progression and survival could take several years to detect significant difference. San Miguel and colleagues have started treating high risk smouldering myeloma patients with lenalidomide and Dexamethasone in a clinical trial. Although early the drug has been well tolerated with some complete responses observed [9]. Since the widespread use of recently licensed therapies thalidomide, bortezomib and lenalidomide, the median overall survival of patients treated upfront or at relapse has significantly increased [10]. In the total therapy 3 protocol which is a dose intense protocol with thalidomide and bortezomib induction followed by tandem autologous transplantation, complete response rates at 24 months in excess of 90% were observed [11]. But despite this impressive advance in treatment of myeloma, most patients inevitably relapse and treatment of

relapsed myeloma remains an area of active investigation. There is no clear consensus on induction or relapse therapy, among the treating physicians. Majority of physicians favour the sequential use of active therapeutic agents with consolidation with high dose therapy. But there are trials combining active agents together for front line use such as Bortezomib, lenalidomide and dexamethasone (RVD)[12] and bortezomib, lenalidomide, thalidomide and dexamethasone (EVOLUTION trial)[13], which have yielded VGPR rates of 67% in a highly selected group of patients confirming significant clinical activity. But it remains to be seen if the responses are durable and whether these patients are salvaged when they progress. In the UK, where PCT funding is guided by NICE appraisal process, by default bortezomib is used for treatment of myeloma patients in first relapse and lenalidomide for subsequent relapses, with almost all patients treated upfront with thalidomide based therapy. Currently there are more than 30 agents in Phase I/II trials either alone or in combination for treatment of relapsed/refractory myeloma. These agents induce modest direct cytotoxic effect on plasma cells in vitro. Although the effects of these newer agents on the microenvironment are poorly delineated, the high response rates clearly indicate that the modulation of the marrow microenvironment, which supports the survival and proliferation of the neoplastic plasma cell clone, plays an important role. Recently clonal evolution resulting in drug resistance to therapies has been proposed. This is currently an active area of research. If this were to be proven, this would drive further requirement of improved therapies as the median survival of myeloma patients has improved.

2.1 Evolution of disease and role of the marrow microenvironment

Research on the role of bone marrow microenvironment in disease progression from MGUS/Smouldering myeloma to symptomatic myeloma is focussed on several major themes. Genetic events, progressive angiogenesis, bone disease and release of soluble factors, adhesion leading to drug resistance and breakdown of immune surveillance are the main areas. For purposes of description in this thesis, the bone marrow microenvironment encompasses haematopoietic and non haematopoietic cells, extracellular matrix and liquid parts of microenvironment (Fig 1)

Figure 1: Description of Bone marrow compartments. The bone marrow is divided into cellular, non cellular and liquid compartments as listed above. Within the cellular compartments both cells of haemopoietic lineage and non haemopoietic lineage are present. The non haemopoietic cellular compartment is loosely termed as marrow accessory cells or cells in the microenvironment. Bone marrow plasma contains the cytokines listed in the liquid compartment of the bone marrow. CEP – circulating endothelial progenitors, HSC – haemopoietic stem cell, ECM- extracellular matrix, PG – proteoglycan, GSG – glycosaminoglycans. Adapted from Podar et al Leukemia (2009) 23, 10–24



2.1.1 Acquired chromosomal changes and gene expression in plasma cells

Progression from MGUS and asymptomatic myeloma to symptomatic myeloma has been an intense subject of research over the last decade. One of the largest cytogenetics studies, where fluorescence *in situ* hybridisation (FISH) analysis was carried out in purified bone marrow plasma cells of 1064 patients recruited in IFM99 trials demonstrated an acquired genomic aberration in 90% of patients[14]. Kuehl and Bergsagel described the oncogenic events during transformation from MGUS to Myeloma. Hyperdiploidy and translocations causing activation of cyclin D1, cyclin D2 or cyclin D3 and deletion 13 are early oncogenic events. In the cells harbouring the translocations with activated cyclins, cyclin dysregulation makes the cells susceptible to proliferative stimuli and enhances interaction with stromal cells. *Myc* translocation is rare event in MGUS but more frequent in early and advanced myeloma with almost all human myeloma cell lines (HMCL) harbouring this feature (Fig.2). This observation confirms the hypothesis that these are secondary events. Other events such as activating mutations of *NRAS*, *KRAS*, *FGFR3* and mutations or deletions in *TP53* are secondary events occurring during disease progression [15]. Gene expression studies performed in CD138 enriched plasma cells by Shaughnessy et al, in 245 newly diagnosed myeloma patients, identified 7 distinct subsets (Fig 3) [16]. They are hyperdiploid sub group, proliferative signature, Low bone disease group, MMSET group, MAF sub group, *CCND1* activation and *CCND3* activation subgroups. Taking the clinical features together with the gene

expression they have classified 7 cohorts of patients, with some genomic subsets having significantly better event free and overall survival compared to others. Gene expression studies comparing normal plasma cells, MGUS and Myeloma plasma cells shows differential expression of genes. The Genes involved in cell signalling (*NFkB*), oncogenes (*RB1*), developmental genes (Wnt pathway) and transcription factor genes were significantly different [17]. Although the level of cyclin expression observed in neoplastic plasma cells is similar to dividing B cells, myeloma cells which are fully differentiated B cells have a very low proliferative rate. The role of the marrow microenvironment providing the matrix and soluble factors necessary to drive the proliferation of these cells within the bone marrow and giving them a survival advantage is key to disease progression. Taken together, acquired chromosomal changes leading to differential expression of genes is involved in progression of disease and leads to clinical heterogeneity.

Fig 2: Genomic changes leading to disease progression: This is a model of sequential genomic events leading to disease progression from MGUS to myeloma. TLC – translocation. Adapted from Bergsagel, P. L. et al. J Clin Oncol; 23:6333-6338 (2005)

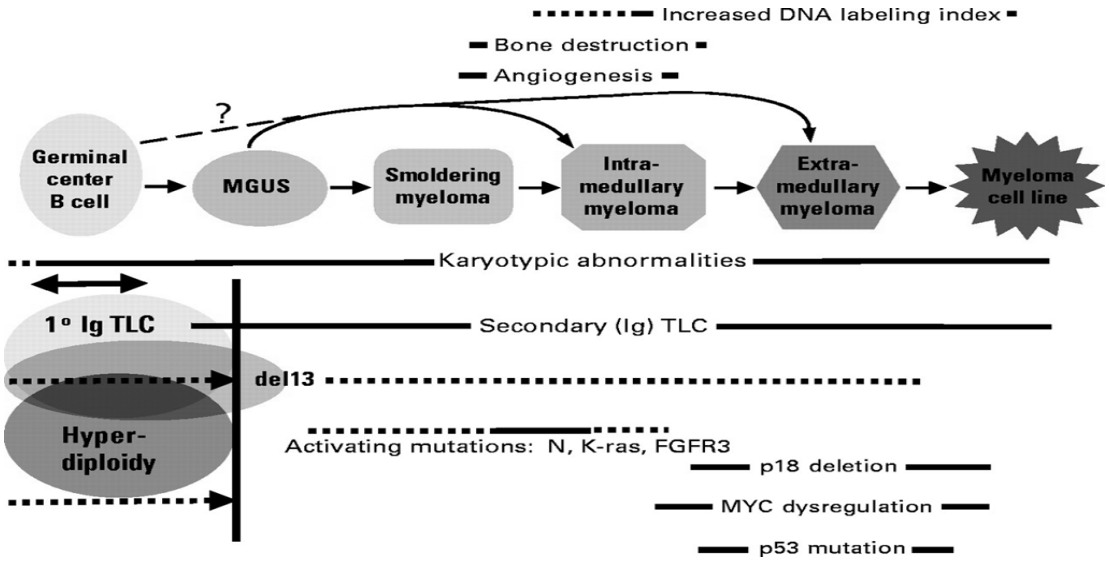
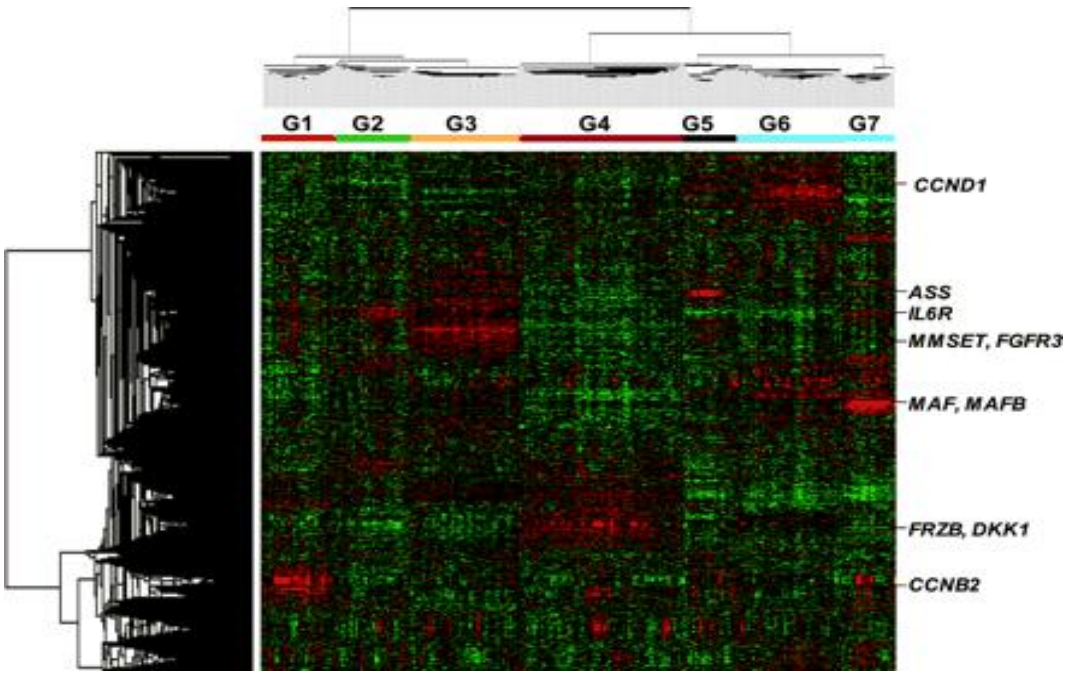


Fig 3: Gene expression profiling in myeloma plasma cells: Gene expression analysis was performed from mRNA extracted from CD138 selected plasma cells from 256 newly diagnosed myeloma patients. The image shows a two-dimensional unsupervised hierarchic cluster analysis of 1559 highly variable genes (rows) in 256 cases (columns). Red and green indicate overexpressed and underexpressed genes, respectively. The sample dendrogram at the top and gene dendrogram to the side reflect the relatedness of the samples. Note that the dendrogram branches are strongly influenced by noticeable clusters of overexpressed genes. Subgroups G1 to G7, from left to right, are indicated under the dendrogram. Adapted from Zhan, F. et al. Blood (2006); 108: 2020-2028



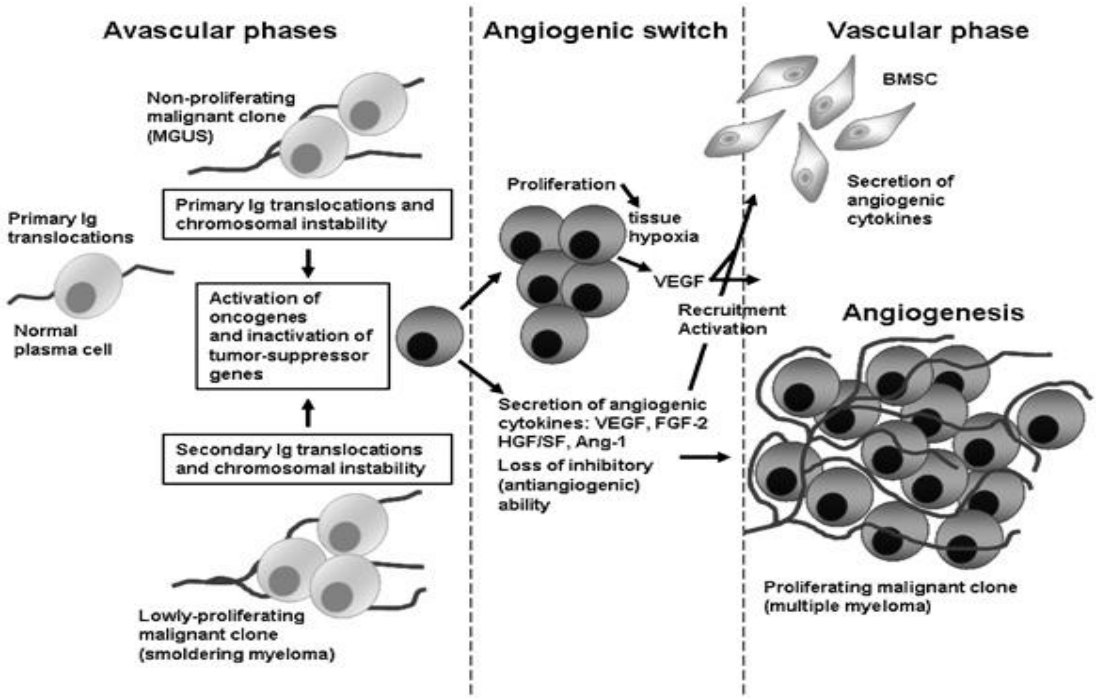
2.1.2 Increased Angiogenesis and the angiogenic switch

Angiogenesis in the bone marrow of myeloma patients is mediated by plasma cells, stromal cells and endothelial cells in the microenvironment. The impact of angiogenesis was evidently observed when bone marrow biopsies of MGUS, early and advanced myeloma were stained with FVIII, a coagulation factor, showing significantly increased microvascular density in relapsed myeloma[18]. Vascular endothelial growth factor (VEGF) secreted by plasma cells acts on cognate receptors on stromal cells thereby releasing soluble factors driving plasma cell proliferation in a paracrine manner [19]. VEGF also directly acts on endothelial cells inducing proliferation and therefore angiogenesis. This results in enhanced survival, endothelial cell permeability and endothelial cell migration. A similar mechanism is suggested in Angiopoietin -1 and Tie -2 receptor interaction [20]. Endothelial cells from myeloma patients cultured for long periods (MMEC) show upregulation of angiogenic genes and receptors, inducing an intense angiogenic response in chick embryo chorioallantoic membrane, in comparison to endothelial cells from MGUS patients [21]. The process of an angiogenic switch causing progression from a relatively avascular phase to a vascular phase is preceded by losing CD45 expression on plasma cells and secretion of high levels of VEGF [22]. It is also hypothesised that there is loss of inhibition of angiogenesis resulting in observed increase in microvascular density (Fig 4).

Tumour associated macrophages secrete proangiogenic cytokines and growth factors and angiogenesis modulating enzymes such as metalloproteinases [23]. Presence of macrophages at a frequency of 2-12% is a frequent occurrence in

plasmacytomas as has been described before[24]. Vacca et al have also shown that these macrophages in the presence of growth factors such as VEGF, FGF-2 can transform into phenotypic and functional endothelial cells. Vanderkerken et al demonstrated the importance a hypoxic bone marrow environment to enhance engraftment and angiogenesis. They used a hypoxia prodrug as proof of principle which induced apoptosis of syngeneic bone marrow plasma cells in 5T33 mice with reduction of tumour load [25]. Finally, thalidomide an antiangiogenic drug significantly reduced microvascular density in bone marrows of responding patients, but no reduction in angiogenesis was observed in non responding patients [26]. In summary, angiogenesis is an active process linked to disease progression in myeloma. This also makes angiogenesis an important target in the microenvironment.

Fig 4: Correlation of angiogenesis with disease progression – Hypothesis: Increased angiogenesis is observed in marrow pf patients with disease progression. This image depicts the changes which lead to angiogenic switch from avascular to a vascular phase. VEGF- vascular endothelial growth factor, FGF – fibroblast growth factor , HGF – hepatocyte growth factor, Ang-1 – Angiopoietin -1, BMSC – bone marrow stromal cell. Adapted from Vacca et al. Leukemia (2006) 20, 193–199



2.1.3 Breakdown of Immune surveillance in the microenvironment

Antigenic targets of spontaneous immunity have been shown to be altered from MGUS to myeloma. MGUS patients mount a humoral and cellular immune response against *SOX2* gene which is important for embryonal cell renewal[27]. Both CD4 and CD8 T cell responses were present in patients with MGUS but not in patients with myeloma. *SOX2* expression was found in a CD138 neg CD19 neg compartment of bone marrow cells, and patients with *SOX2* specific T cells had a significantly lower rate of disease progression compared to patients lacking the T cell response. The Idiotype protein is a myeloma restricted antigen but idiotype vaccinations have not induced significant clinical responses in myeloma patients[28]. Humoral and cellular immune response against *OFD1* a centrosome related protein, which plays a role in Wnt and Hedgehog signalling pathway in myeloma patients, was detected in both MGUS and myeloma patient samples. Downregulation of *OFD1* using siRNA in MM1S cells downregulated the proteins involved in the pathway[29]. Cancer Testis antigens are also aberrantly expressed by myeloma cells and are a potential immunotherapeutic target. CD4+ T cell immunity and cytotoxicity was observed more in MGUS than in MM patients against melanoma associated antigen (MAGE) positive myeloma cell lines. CD8+ memory T cell responses were seen exclusively in myeloma patients but these cells were poorly recruited into the bone marrow[30].

Increased frequency of naturally occurring functional CD4(+)CD25(+)FoxP3(+) T(Reg) cells in patients with MM as well as MGUS in comparison to age-matched, healthy controls has been reported. The exact

mechanisms through which they modulate the immune function in myeloma and whether they play a role in disease progression, remains unclear[31]. Expression of MIC-A and NKG2D in NK cells of myeloma patients were lower in comparison to MGUS patients and these findings could potentially be linked to disease progression[32]. Recent studies looking at aberrant antigen processing machinery in myeloma patients demonstrated reduction in expression of proteasome subunits and peptide transporters. RT-PCR experiments showed changes in antigen processing machinery at the transcriptional level. These results allude to antigen processing aberration as one of the mechanisms of impaired immune surveillance in myeloma patients[33].

On a more clinically relevant basis, thalidomide and lenalidomide induce natural killer (NK) cell activation. IMiDs induce IL-2 transcription and secretion by activation of PI3kinase activation which induces NK cell proliferation and activation[34]. Dhodapkar et al showed lenalidomide and dexamethasone in combination induced natural killer T cell (NKT) expansion and activation in vitro and in vivo in myeloma patients in the presence of NKT ligand. This serves as an alternative mechanism for NK activation [35]. These observations and experiments show a defective immune response and defective immune surveillance in myeloma patients. Some of the early therapeutic results are encouraging and could potentially lead to immunotherapy trials either with cell therapy or with drugs such as IMiDs.

2.2 Evolution of myeloma therapy

The malignant plasma cells in myeloma, build up through a combination of increased proliferation and abnormal cell survival. Inhibition of normal cell death can occur in response to both intracellular and extracellular signals and is mediated by a complex series of cellular processes in the plasma cells. Conventional chemotherapy agents interfere with the synthesis of DNA or the process of cell division. Alkylating agents, anthracyclines and corticosteroids have been the mainstay of myeloma therapy until the start of this decade. Melphalan was the first chemotherapy agent to be used to treat myeloma and cyclophosphamide, a non-cross resistant alkylating agent, has also been shown to be effective and has the added benefit of being less myelotoxic. This is particularly advantageous in treating patients who have received previous chemotherapy and in elderly patients with reduced bone marrow reserve. Anthracyclines have significant cardiotoxic and myelosuppressive effects and were commonly used in combination with corticosteroids. The poor responses reported with chemotherapy are primarily due to the increased incidence of drug resistance that occurs with successive lines of therapy and also because of cumulative myelotoxicity and organ toxicity, which restricts the intensity of drug delivery. Dexamethasone remains an effective agent in the newly diagnosed and relapsed settings because it is not excreted by the kidney and so can be given in the face of renal impairment. However, responses are short lived with a median of only 4 to 6 months in most studies. Long term steroid therapy increases risk of infection, gastrointestinal toxicity and osteoporosis.

2.2.1 Thalidomide and its analogues (IMiDs)

Over the last five years, three novel agents have been licensed to treat this disease. Thalidomide, Bortezomib and Lenalidomide were licensed on the back of Phase III trials recruiting significant number of patients. Thalidomide and its analogues and proteasomal inhibitors represent new classes of active drugs against myeloma in over two decades. Thalidomide role as an antiangiogenic agent was identified in 1994 and its activity in myeloma was confirmed in a phase II study[36, 37]. Thalidomide has antiproliferative, proapoptotic effects on myeloma cell lines and primary cells from myeloma patients[38]. These effects were enhanced and synergistic with addition of dexamethasone to the cultures. Thalidomide apart from being antiangiogenic possesses a strong anti-inflammatory property with significant reduction in *TNFα* levels *in vitro*. Thalidomide also has an immunomodulatory role by enhancing T cell proliferation and NK cell cytolytic activity *in vitro*[39]. A large phase III study in newly diagnosed patients confirmed the results obtained in smaller phase II studies, and further demonstrated a durable response with improved time to disease progression[40]. IMiDs, thalidomide analogues with potent *in vitro* and *in vivo* activity, of which Lenalidomide (Revlimid®, Celgene Corporation) and pomalidomide (Actimid®, Celgene Corporation) are extensively studied and are in clinical use for myeloma. Hideshima et al and Davies et al in their experiments clearly demonstrated that these analogues have much more potent antiproliferative, proapoptotic and immunomodulatory effects with activation of T cell and NK cell response [38,39]. Thalidomide analogues are also several thousand fold potent than thalidomide in reduction of *TNFα* levels in vitro. Although IMiDs have a direct proapoptotic effect on

resistant myeloma cell lines in vitro, the effects on the microenvironment underpins its clinical activity (Fig.5). Two large Lenalidomide and dexamethasone (Len- Dex) trials at a median follow-up from randomisation of 17.1 months (MM-009) and 16.5 months (MM-010), showed a significant improvement with Len-Dex compared to Dexamethasone in overall response (MM-009: 61% vs 20.5%, p<. 001; MM-010: 59.1% vs. 24%, p<. 001, respectively) and time to progression (TTP) (MM-009: 11.1months vs. 4.7months, p<. 001; MM-010: 11.3months vs. 4.7months, p<.001, respectively [41,42]. The immunomodulatory effects previously alluded to in the thesis makes lenalidomide a useful maintenance agent for the purposes of immunotherapy [35]. Lenalidomide has also been shown to inhibit osteoclastogenesis in vitro, by down regulation of pERK and transcription factor PU.1. The secretion of RANKL from stromal cells in the microenvironment is also significantly reduced. These effects resulted in improvement of osteoprotegerin (OPG) levels in patients treated with lenalidomide [43]. Pomalidomide has similar activity and inhibits osteoclastogenesis by down regulating PU.1 a transcription factor affecting lineage commitment, which translated to inhibition of bone resorption[44].

2.2.2 Proteasomal Inhibition

Bortezomib a dipeptide boronic acid is a potent, highly selective, and reversible proteasome inhibitor that targets 26S proteasome complex and inhibits its function of intracellular degradation of ubiquitinated proteins. Accumulation of misfolded proteins causes heat shock protein activation and induces cell death. Bortezomib has various mechanisms of action against

myeloma cells including induction of apoptosis by caspase 9 activation, inhibition of nuclear factor-κB activation, triggering of caspase-dependent cleavage of gp130, down regulation of adhesion molecule expression on myeloma cells [45]. Bortezomib also down regulates adhesion receptors on BM stromal cells, inhibits angiogenesis and decreases secretion of survival and proliferative cytokines. Up to one third of patient do not respond to bortezomib and a proportion of patients acquire resistance by exposure to the drug. Resistance to bortezomib is mediated through over expression of bcl-2 and Hsp27 [46,47]. There are other possible mechanisms suggested for bortezomib resistance such as MCL-1 activation, high levels of IL-6, proteolytic subunit B mutation and activation of aggresome pathway, which is the other pathway for protein degradation (Fig 6). FDA approval was based on the results of the APEX study showed a highly significant difference between the Bortezomib and High dose Dexamethasone (Dex) arms in relapsed/refractory myeloma patients ($p<0.001$) for TTP, but this only amounted to 3 months difference in progression free survival (PFS) between the 2 arms [48]. Bortezomib inhibits osteoclastic activity and therefore enhances plasma cell apoptosis by reduction in release of soluble factors[49]. Bortezomib also enhances osteoblast differentiation through activation of beta catenin/T cell factor signalling and leads to alteration in the tumour microenvironment of myeloma patients, observed in bone marrow biopsies of treated patients and elevation of serum markers of bone formation[50] [51]. Bortezomib also regulates osteogenesis via the induction of BMP-2 expression, increasing Runx2 transcriptional activity and stabilization of Runx2 protein[52]. Carfilzomib a new irreversible proteasome inhibitor inhibits chymotrypsin like activity of the proteasome. In

myeloma cells, chymotrypsin like inhibition alone was sufficient to induce proapoptotic effect, including proteasome substrate accumulation, *Noxa* and caspase 3/7 induction, and phospho-eIF2alpha suppression[53]. Carfilzomib has shown significant activity in Phase I/II clinical trials in relapsed/refractory myeloma patients.

Although the response rates of relapsed myeloma patients to these novel agents is much higher compared to historical data, patients are still not cured and eventually progress. These agents when used in frontline treatment of myeloma induce much higher response rates but whether this would translate into longer progression free survival remains to be seen. It has become obvious, that bone marrow microenvironment supports survival of myeloma plasma cells and therapies targeting the plasma cell clone and the bone marrow stromal/MM interaction are effective in relapsed disease [54]. Despite these effective new agents, there is still a clearly unmet medical need for novel innovative agents to be developed to treat multiple myeloma.

Fig 5: Effects of Thalidomide and thalidomide analogues (IMiDs) on the myeloma microenvironment. Thalidomide and thalidomide analogues apart from direct effects on tumour cell also modify the microenvironment by its effects on stromal cells and angiogenesis in the marrow. Adapted from Quach et al, *Leukemia* (2010) 24, 22–32

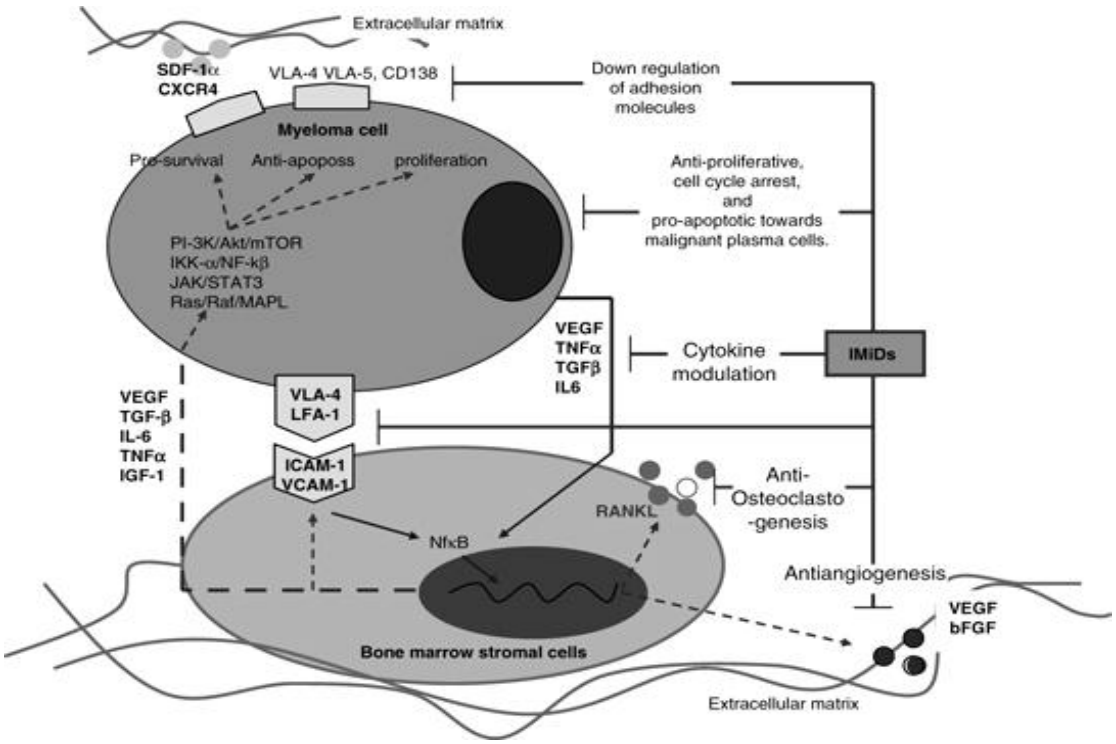
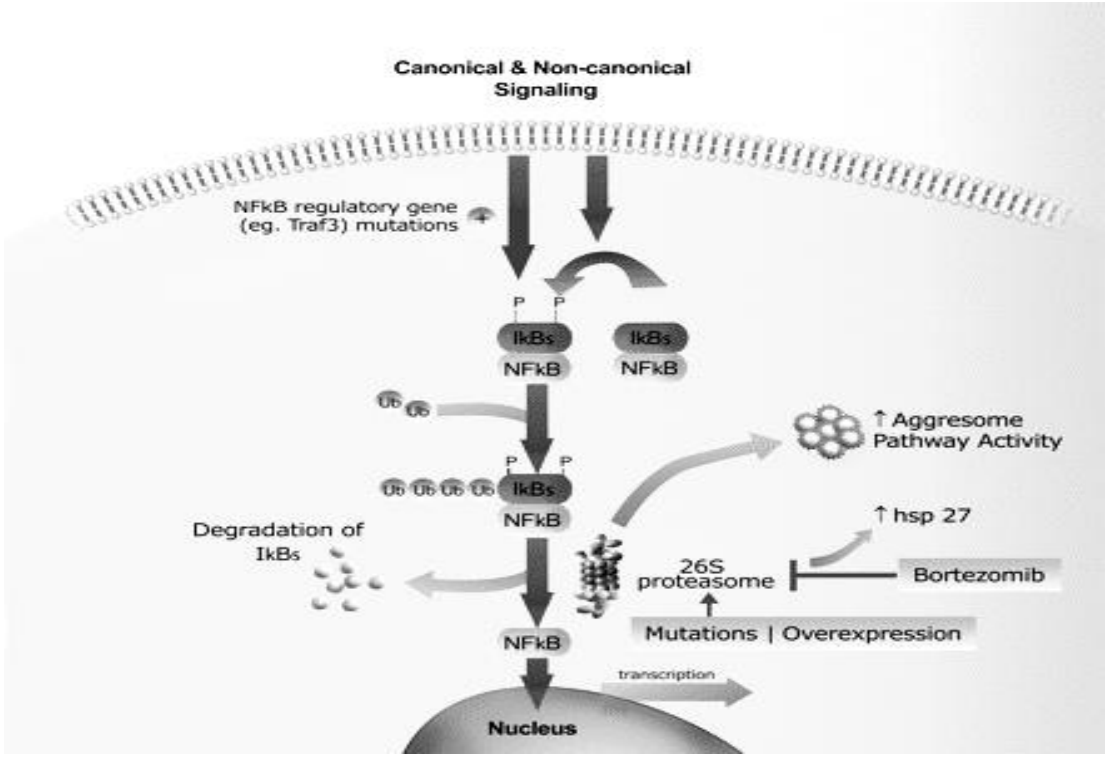


Fig 6: Mechanisms of bortezomib resistance. Bortezomib a proteasomal inhibitor is effective in a majority of myeloma patients. This cartoon depicts the intracellular changes leading to resistance to therapy. Adapted from Kumar, S. et al. Blood 2008; 112:2177-2178



2.3 Primary and acquired resistance in myeloma

Primary resistance to therapy is exhibited as either lack of or suboptimal response to therapy. Patients relapsing from previous therapy also exhibit features of acquired resistance which determines long term survival in these patients. Therapy sensitivity is restored in some patients through synergistic drug combination with targeted action eg; bortezomib. Expression of drug resistance proteins such as P- glycoprotein, which plays an important role in leukaemia survival have been shown to play an important role in chemotherapy resistance observed in myeloma. Soluble mediators and accessory cells in the microenvironment mediate resistance particularly in the relapsed/refractory patients.

2.3.1 Genomic aberrations and primary resistance

Cytogenetic aberrations up regulate oncogenes or down regulate proapoptotic genes resulting in the resistant phenotype observed in some subsets of myeloma. Acquired genomic abnormalities have been identified both in MGUS and in MM patients. Up to 90% of patients have a cytogenetic abnormality in purified myeloma cells obtained from bone marrow FISH[14]. The commonly observed del 13 cytogenetic abnormality is detected in up to 50% of patients on interphase FISH. Detection of del 13 abnormalities can be made by conventional cytogenetics, metaphase FISH and interphase FISH. Presence of del 13 detected by conventional cytogenetics is clearly linked with poor prognosis but not by interphase FISH[55]. Prior to the availability of IMiDs and proteasomal inhibitors, patients treated with combination chemotherapy and consolidated with autologous transplantation had a superior response and

survival if they lacked del 13 abnormality on cytogenetics[56]. In patients recruited in IFM99 trials, the presence of t (4:14), and del (17p) abnormalities on interphase FISH were independently associated with poor event free and overall survival[14]. In a sub analysis from the Bortezomib trials the drug appears to overcome the poor prognosis conferred by del 13[57]. Data now available on patients treated with bortezomib and lenalidomide and the combination in the form a Total Therapy 3 trial approach show responses in patients carrying high risk cytogenetic features[11]. But it still remains to be seen if these patients progress early after therapy. In patients with high risk cytogenetics[58], transactivation of oncogenic kinases or oncogenes such as *FGFR3*, *Myc*, *NRAS*, *KRAS* and dysregulation of cyclin dependent kinases gives cell a proliferative advantage leading to poor response to therapy and early disease progression. It is also hypothesised that patients who progress after prior therapy continue to develop further genomic abnormalities due to survival benefit exhibited by the clone. But there is a school of thought that apart from *TP53* deletion further genomic changes at disease relapses is rare. This will probably be better addressed by performing single nucleotide polymorphism arrays (SNP array) analysis in relapsed patients, to see if further genetic events are acquired with disease progression.

2.3.2 Therapy induced resistance

Expression of MDR1 and its 170-kDa protein product, P-glycoprotein (P-gp), also designated as adenosine triphosphate (ATP)-binding cassette (ABC) transporter B1 (ABCB1) modulates response to chemotherapy. P-gp is a transmembrane protein that acts as an energy-dependent drug efflux pump

for chemotherapeutic drugs. Recent data also shows that C3435T polymorphism of MDR1 influences response to treatment and hence overall survival in uniformly treated myeloma patients[59]. Bortezomib sensitivity in cancer cell lines is regulated by P-gp and MDR1 as evidenced by greater inhibition of the proteasome when the proteins were downregulated with siRNA. Up to one-third of myeloma patients are resistant to up front bortezomib therapy. Although other mechanisms are reported in literature, MDR1 up regulation could be a significant factor in relapsed myeloma patients. Expression of P-gp is significantly increased in relapsed myeloma patients and is associated with resistance to doxorubicin[60]. Addition of verapamil and cyclosporin to modulate P-gp expression in patients on myeloma therapy has shown little clinical benefit, with potential additive toxicity[61]. Therapy resistance may also be mediated by other multidrug resistance proteins. Multidrug-resistance associated protein, also known as ATP-binding cassette, subfamily C, member 1 (MRP1, or ABCC1) is also expressed in myeloma cells. LRP (lung resistance protein) is present in myeloma cells of newly diagnosed patients. Response to melphalan therapy is lower in myeloma patients with LRP expression compared to those who have no LRP expression[62]. Taken together, expression of MRD1, MRP1 and LRP play an important role in mediating response to therapeutic agents. Newer agents in development should be tested on myeloma cell lines expressing these proteins.

2.3.3 Resistance to glucocorticoid therapy

Glucocorticoids have been the mainstay of treatment for myeloma for a number of years. High dose dexamethasone remains a standard comparator in major phase III trials. Brisk early responses are seen with steroids which is particularly useful when patients present with cord compression or renal failure. Over the course of their disease, myeloma patients become resistant and refractory to glucocorticoids. Glucocorticoid resistance in myeloma has been shown to be due to post receptor defects in the downstream signalling pathways. Recent data also suggests that decrease in expression of isoform of glucocorticoid receptor, GR alpha can mediate resistance in myeloma cell lines [63]. A recent study investigated if epigenetic modification of DNA correlated with dexamethasone resistance in a human myeloma cell line OPM1. Epigenetic inactivation of RASD1 which encodes a ras family protein was found to be correlated with dexamethasone resistance[64]. Although glucocorticoid induced changes within the plasma cell has been well studied, changes in the microenvironment is less well understood.

2.3.4 Role of microenvironment in induction of drug resistance

Homing of tumour cells to the bone marrow through the SDF-1/CXCR4 signalling pathway plays an important role in creating protective niches for plasma cell survival within the bone marrow[65]. Bone marrow is a rich source of cytokines and growth factors required for survival and proliferation of progenitor haematopoietic cells, which is utilised by neoplastic tumour cells when they home to the marrow. Interleukin-6 (IL-6) is an important growth

factor for multiple myeloma cells and induces *STAT3* signalling that confers protection from Fas-mediated apoptosis by up-regulating the antiapoptotic protein Bcl-XL in myeloma cells[66]. Primary samples producing IL-6 were found to be resistant to both spontaneous and dexamethasone induced apoptosis, whereas non–IL-6 secreting clones were sensitive[67]. Monoclonal antibody CNTO-328 directed against IL-6 receptor showed blockage of IL-6 signalling in myeloma cell lines and increased their sensitivity to bortezomib[68]. This observation is currently being studied in a prospective randomised clinical trial. Bone marrow stromal cells produce high levels of IL-6 and paracrine interaction of myeloma cells with stromal cells through soluble intermediaries enhances the secretion of growth factors by both cell types. Primary myeloma cells also produce vascular endothelial growth factor and fibroblast growth factor which, in turn, stimulates IL-6 production by stromal cells. Adhesion of myeloma cells in the microenvironment also induces acquired resistance against chemotherapeutic agents. This is discussed in detail in section 2.4. Osteoclasts are activated by cytokines released by stromal cells and plasma cells. The role of osteoclasts in promoting the survival and proliferation of plasma cells in patient samples has been elegantly described by two groups. This effect requires cell-cell contact between plasma cells and osteoclasts [49,69]. These features put together demonstrate that there are multiple factors governing resistance in myeloma cells. Some of the factors are intrinsic to the property of tumour cells and modulated through exposure to drugs but resistance is also mediated by accessory cells in the marrow and soluble factors in the microenvironment. This complex situation leads to inevitable

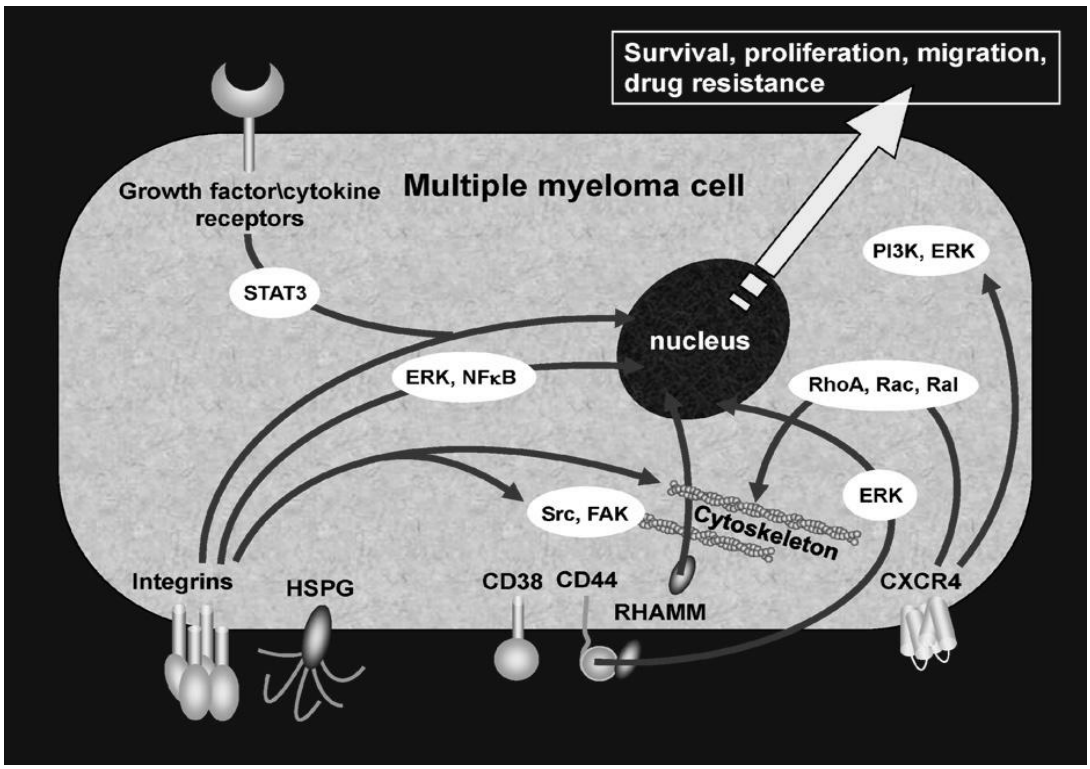
relapse of disease when patients are followed up, and the biological nature of the cells at relapse makes therapeutic options limited with variable success.

2.4 Myeloma cell adhesion and cell adhesion mediated drug resistance (CAM-DR)

Tissue formation in normal physiology requires cell adhesion. There are two types of cell adhesion, cell-cell adhesion and cell – extra cellular matrix (ECM) adhesion. Tight junctions, Adherens junctions and desmosomes are the three types of junctions which mediate adhesion between epithelial cells. Cell adhesion involves membrane anchors, transmembrane receptors, extracellular ligands and cytoskeketal components. Cadherins, transmembrane receptors constitute a large family of glycoproteins comprising an extracellular domain responsible for cell-cell interactions, a transmembrane domain, and a cytoplasmic domain that frequently is linked to the actin cytoskeleton. Cadherins are found in both adherens junction and desmosomes and play a key role in calcium-dependent cell-cell interaction [70]. Cell–matrix contacts are specialised zones at the cell surface, where activated or clustered adhesion receptors bind to their ECM ligands and link intracellularly to components of the cytoskeleton. Cell-matrix contacts can exist as focal complexes, podosomes, focal adhesions, fibrillar adhesions, three dimensional matrix adhesions and hemidesmosomes. Best characterised of these are the focal adhesions[71]. There are five classes of cell adhesion molecules (CAM) – integrins, cadherins, immunoglobulin-like CAMs, selectins and proteoglycans. They have all been shown to be deregulated in human cancer. Integrins and syndecan-1/CD138 are the principal myeloma receptor systems of extracellular matrix components, as well as of surface molecules of stromal cells. CD44 and receptor for hyaluronan-mediated motility (RHAMM) are the major hyaluronan receptors and fibronectin and

osteopontin are chief ligands of integrins expressed on myeloma cells (Fig 7)[72].

Fig 7: Myeloma cell adhesion and signalling pathways. Myeloma cell interacts with the microenvironment through different sets of adhesion molecules as shown here leading to activation of intracellular pathways. Adapted from Katz et al Seminars in cancer biology Epub Apr 2010

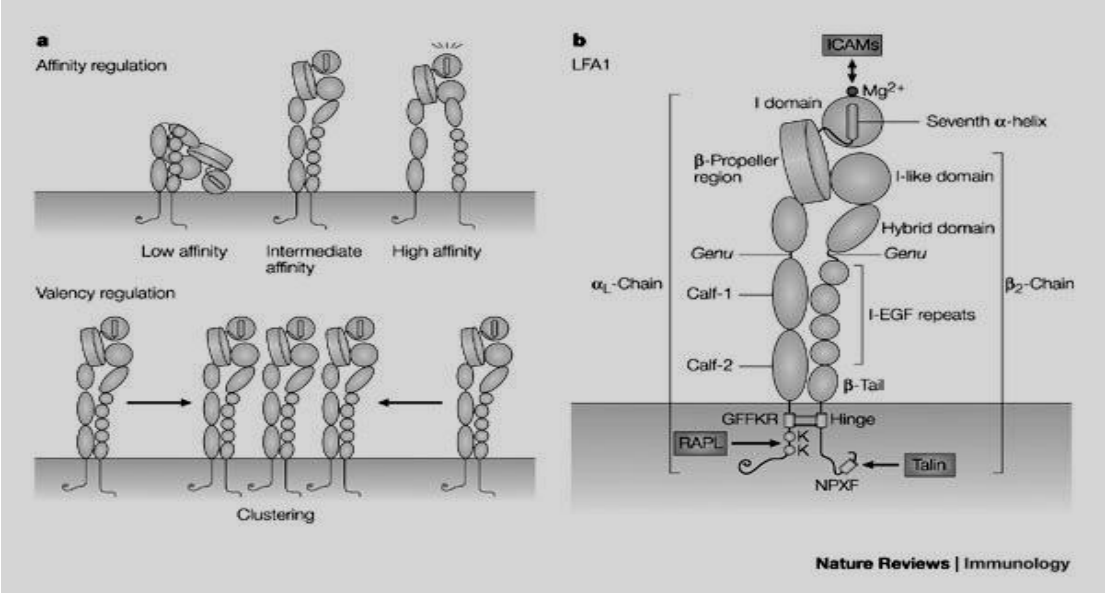


2.4.1 Integrin mediated adhesion

Integrin class of cell adhesion molecules are α/β heterodimeric glycoprotein receptors that regulate cellular processes such as cell migration, phagocytosis and growth and development. Integrins recognise extracellular matrix proteins and cognate receptors on other cells and following ligand binding can activate or contribute to the same cellular signalling pathways that are regulated by growth factor receptors. They are always composed of two subunits, α and β. To date 18 α and 8 β subunit isoforms forming 24 combinations have been identified. Each α β combination has its own binding specificity and signalling properties. A specific ECM molecule can nevertheless be bound by different types of integrins, and specific integrins can bind to different types of ECM molecules. Integrin-mediated adhesion to ECM ligands such as fibronectin, collagen, vitronectin, osteopontin and laminin is mediated by recognition of arginine – glycine - aspartic acid (RGD) motifs in these proteins [73]. Integrins can signal bidirectionally; interaction with ligand/external stimuli to induce intracellular changes (outside-in signalling) and intracellular stimuli can also cause extracellular changes (inside-out signalling). Integrin activation increases the affinity of individual integrins for ECM ligands, to bind strongly to ECM requires increasing the avidity of the interaction by clustering integrins (Fig.8) [74]. In myeloma, adhesion of plasma cells to extracellular matrix proteins such as fibronectin, laminin, collagen and oseopontin in the microenvironment is mediated by integrins. Integrins are also important in the tumour pathogenesis of myeloma and surface expression of cell adhesion molecules analysed by flow cytometry varies between progressive disease and stable disease[75]. Recently it has been shown by immunohistochemistry

that CD49d and CD49f expression is significantly higher in plasma cells present in the bone marrow compared to being in a solitary bone location[76]. α5 β1 integrin (CD49e or VLA-5) and α4 β1 integrin (CD49d or VLA-4) interact with fibronectin. Both are expressed in both normal and malignant plasma cells [77]. Cytokines such as TNFα, Hepatocyte growth factor (HGF), Insulin growth factor -1(IGF-1) increased in bone marrow plasma of these patients activate integrins. $\alpha_E\beta_7$ mediates adhesion of plasma cells on stromal cells interacting with E-cadherin [78]. $\alpha_v\beta_3$ integrin is over expressed in patient with skeletal disease and is thought to mediate osteoclast activation by interacting with vitronectin and fibronectin [79]. Engagement of integrins with their ligands such as vascular cell adhesion molecule-1 induces signalling critical for the regulation of proliferation, migration, and survival of normal haematopoietic and epithelial cells. Talin a cytoskeletal protein plays a central role in integrin activation by interacting with β integrin tail. Members of kindlin family are also involved in activation of integrins [80]. Integrin activation leads to conformational change, clustering and recruitment of other cytoskeletal proteins such as paxillin and vinculin. Focal Adhesion Kinase (FAK) generates docking sites for SH2-domain-containing proteins; including Src kinases, which in turn become activated and phosphorylate FAK. This leads to downstream activation of Ras/Raf/MAPK signalling pathways. Taken together, integrins play an important role on adhesion of plasma cells to the microenvironment. This makes integrins a potential target for cancer therapeutics [81,82]

Fig 8: Integrin affinity, clustering and LFA-1. Integrin state, level of affinity and activation leading to clustering is shown in panel A. Panel B shows structure of LFA-1 (lymphocyte function associated antigen -1) which is a dimer of α and β chains. ICAM – Intercellular adhesion molecule, RAPL – GTPase RAP1 effector). Adapted from Kinashi et al Nat Rev Immunol 5, 546-559 (July 2005)



2.4.2 Heparan sulphate proteoglycans and CD44 mediated adhesion

CD138, syndecan -1 is a specific marker for plasma cells. Syndecan is a transmembrane heparan sulphate proteoglycan (heavily glycosylated glycoprotein) which can be shed in the marrow microenvironment and also when cells are manipulated ex vivo. Syndecans act as receptors for ECM proteins and for growth factors. They engage through the large flexible glycosaminoglycan chains that make them ideal receptors for ligands that are distant from the membrane. The short cytoplasmic domains can be subdivided into two conserved regions that bind Src/Fyn Tyr kinase complexes and PDZ-domain-containing proteins, respectively, and a central variable region [83]. Focal adhesion formation when cells are plated on fibronectin is dependent on integrin engagement and cooperation with a cell surface proteoglycan like syndecan. It is also thought that Syndecan -1 cooperates with $\alpha_v\beta_3$ integrin and causes pathological neovascularisation. This could be linked with the increased microvascular density observed in these patients with relapsed myeloma with significant bone marrow plasmacytosis. The presence of heparanase in the microenvironment cleaves and activates the shed heparan sulphate releasing sequestered growth factors and triggering clustering of syndecan (activation) and promoting tumour growth. Yang and colleagues tested three different methods to down regulate syndecan-1 (CD138) effects; by adding heparinase, down regulating heparanase and an antibody targeting syndecan-1. All these strategies down regulated syndecan expression and led to tumour regression in vivo in a mouse model [84]. Antibodies against syndecan-1 expressed on myeloma cells by a conjugate of cytotoxic compound and anti-CD138/maytansinoid significantly inhibited growth of

myeloma cells, and blocked adhesion of myeloma cells to marrow stromal cells and induced tumour regression in vivo in SCID–Hu mice. Heparanase expression can also be down regulated by Defibrotide a polydispersible oligonucleotide, which has very little cytotoxic activity on myeloma cell lines in vitro. But in mouse models, defibrotide sensitised myeloma cells to other cytotoxics, reduced adhesion and angiogenesis leading to tumour regression[85].

CD44 splice variants, RHAMM and CD168 are receptors for the abundant amount of hyaluronan present in the microenvironment produced by stromal cells and haematopoietic progenitors in myeloma patients. Normal CD44 expression is down regulated in myeloma cells and higher expression of cytoplasmic RHAMM noted in myeloma cells is a marker of genomic instability. There are a number of publications correlating the presence of splice variants of CD44 in patients and their poor prognostic disease [86,87]. IL-6 which drives myeloma cell proliferation also up regulates CD44 gene expression and alternative splicing of CD44. This generates CD44 splice variants seen in neoplastic plasma cells [88]. Adhesion through CD44 variants expressed on myeloma cell lines to hyaluronan induces resistance to drugs such as dexamethasone [89]. Addition of Bivatuzumab a monoclonal antibody specifically targeting CD44v6 which is a splice variant, reduced adhesion of myeloma cells to stromal cells. This drug also shows promising in results in other solid tumours [90]. Highly immunogenic CD8(+) T-cell epitope peptide derived from RHAMM was used in a phase 1 peptide vaccination programme in patients with MDS and myeloma. This trial has generated encouraging results[91]. Aurora kinase inhibitors appear to produce significant responses in

myeloma patients with higher expression of RHAMM. These drugs are currently in Phase I clinical trials.

2.4.3 Cell adhesion mediated drug resistance (CAM –DR)

Drug resistance induced by adhesion to ECM plays an important role in the pathological manifestations of myeloma. The term cell adhesion mediated drug resistance (CAM-DR) was coined in studies by Damiano and Dalton et al in MM cells, and is now extended to other types of cancer, as part of environment-mediated drug resistance [77,92]. CAM-DR to a variety of drugs such as melphalan and steroids in myeloma cells can be induced by FN or BM stromal cells and is mediated by integrins, predominantly by α4 β1. Damiano et al also showed, selecting for drug resistant myeloma cells leads to the up-regulation of the integrins VLA-4 and VLA-5. Drug-sensitive myeloma cells when adhered to FN demonstrated a reversible de novo drug resistance phenotype that was not due to reduced drug accumulation in cells or up-regulation of bcl-2 family members [77]. The same group demonstrated that upon adhesion on FN, cells are arrested in G1 phase with up regulation of $p27^{kip1}$, which reversed on disruption of adhesion with cells moving into S phase. $p27^{kip1}$ antisense oligonucleotides did not affect adhesion on FN, but $p27^{Kip1}$ protein levels were reduced and cells became chemosensitive [92]. Heat Shock Protein 70 is also up-regulated in myeloma cells and may be associated with CAM-DR. NFkB pathway activation was also demonstrated to be when myeloma cells adhered to FN, and may possibly play a role in CAM-DR featured in myeloma [93]. The resistance exhibited during CAM-DR is not

associated with reduction in drug-induced DNA damage, but rather in protection from mitochondrial perturbations and caspase activation[94].

Myeloma cells when adhered to stromal cells are resistant to commonly used agents such as bortezomib, doxorubicin, melphalan and Dexamethasone [95 94]. Targeting the integrins using monoclonal antibodies restored partial sensitivity to these agents. But targeting the HMG-CoA/GG-PP/Rho/Rho-kinase pathway using statins restored almost complete chemosensitivity when myeloma cells were plated on stromal cells[96]. Wnt signalling pathways, the Wnt/β-catenin pathway (canonical pathway) and the Wnt/RhoA pathway (non-canonical pathway), are implicated in plasma cell migration and bone disease. Wnt / RhoA / ROCK pathway has been shown to modulate CAM-DR of plasma cell adhesion on stromal cells through VLA-6 receptor[97].

Some of the integrin-mediated responses of MM cells are in fact the outcome of a convergence of biochemical signals originating from adhesion molecules and growth factor\cytokine receptors. Intracellularly, upon engagement of integrin with fibronectin, IL-6 mediated STAT3 phosphorylation, nuclear translocation, and DNA binding ensue. Fibronectin facilitates the association of STAT3 and gp130, a part of the IL-6 signalling complex. These form potential kinase targets which can be exploited by therapy. With disease progression myeloma cells activate integrins and interact with the matrix proteins and stromal cells within the microenvironment leading to poor response to therapies. Lymphocyte function-associated antigen 1 (LFA-1) an integrin receptor constitutively activates the FAK/PI3-K/Akt survival pathway in myeloma and targeting this pathway by LFA-1 inhibitors, inhibitors of the Src family kinases, and PI3-K inhibitors induces apoptosis in myeloma cell lines

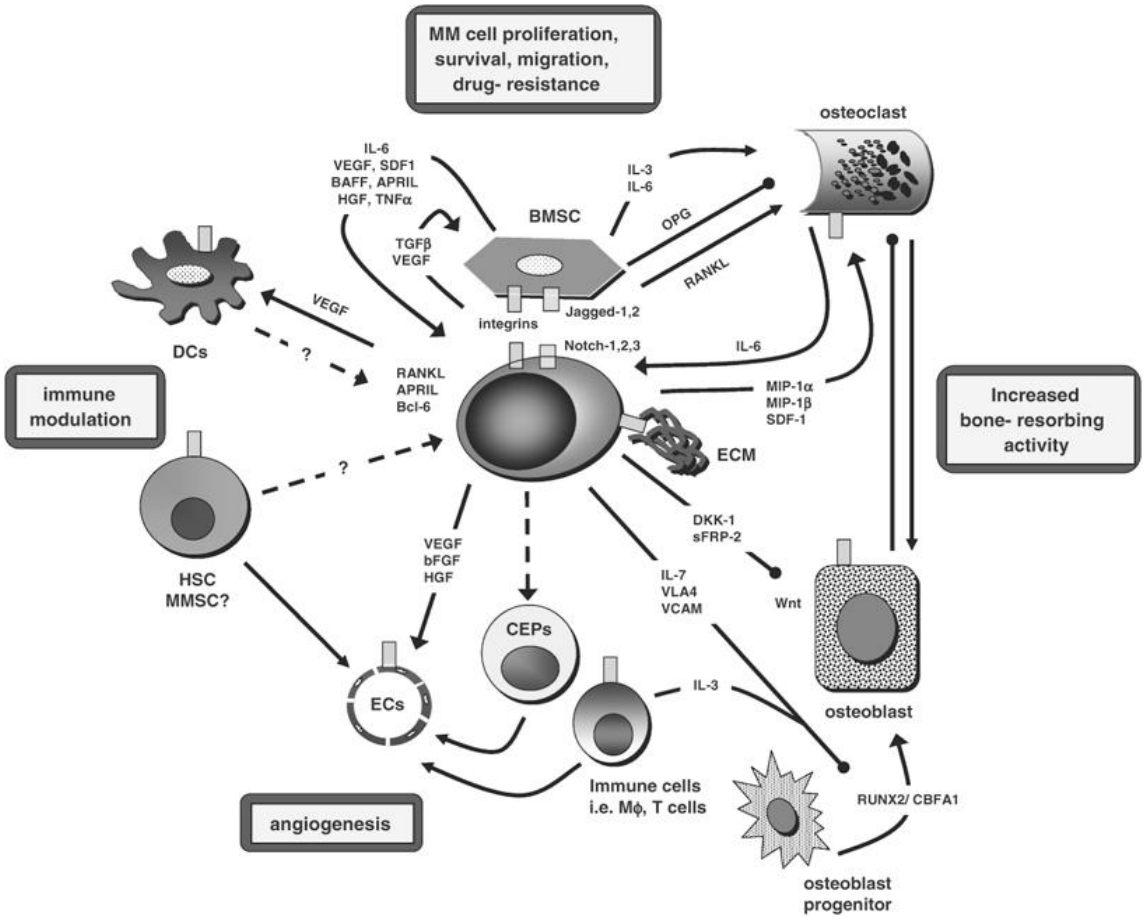
and primary myeloma cells [98]. In summary, CAM-DR is a key mechanism by which myeloma cells exhibit inertness to current day therapy. CAM-DR has been subsequently demonstrated in other haematologic malignancies and solid tumours. It remains to be seen if non chemotherapeutic targeted agents will be able to overcome some of these effects.

2.4.4 Therapeutic targets in the microenvironment

Primary cells from patients cannot be maintained in long term cultures ex vivo. Direct and indirect interactions of plasma cells with other cells and the ECM within the BM microenvironment are key requirements for myeloma pathogenesis, cell growth, survival, migration and drug resistance (Fig 9). Cytokines and growth factors that are produced and secreted within BM microenvironment regulate both by autocrine as well as paracrine loops interaction between plasma cells and other cells. IL-6 is a key growth and survival factor in myeloma, predominantly produced and secreted by stromal cells and osteoblasts. IL-6 triggers activation of MEK/MAPK-, JAK/STAT3, and PI3K/Akt-signalling pathways. Compounds targeting IL-6 signalling pathways include antibodies against IL-6 and IL-6 receptor, CNTO 328, IL-6 antisense oligonucleotides and IL-6 super antagonist Sant7[99]. VEGF is secreted by several myeloma cell lines and present in patient plasma. VEGFR-1 is highly expressed by MM cells, consistent with autocrine signalling. In addition to bevacizumab a VEGF-A antibody, a multitude of other VEGF-targeting compounds are under preclinical and clinical investigation in MM including pazopanib, sorafenib and sunitinib. CD40 is expressed by antigen-presenting cells, T cells, as well as myeloma cells. Functionally, it mediates p53-dependent increase in MM cell growth. Targeting CD40-signalling in MM cells inhibits monoclonal Ig secretion and overcomes CAM-DR. Tumour cells and stromal cells express high levels of BAFF (B cell activating factor) and APRIL (a proliferation inducing ligand), as well as their receptors. Preclinical data suggest therapeutic value of targeting BAFF/APRIL-induced signalling pathways [100]. In summary, numerous

therapeutic targets are being studied in the microenvironment particularly, soluble factors, growth receptor pathways and deregulated kinases using antibodies and small molecules. Agents targeting adhesion of plasma cells will combine theoretically well with cytotoxic agents. Small molecule inhibitors to integrins have been developed and VLA-4 has been a target used more frequently in models of asthma. Ghobrial et al have shown this as a proof of principle in mouse models using CXCR4 inhibitor in combination with anti myeloma agents[95]. Reduction in the adhesion of so called resistant plasma cells would enhance remissions but these adhesion interactions also anchor stem cells and myeloid cells in the marrow[101]. Therefore haematological toxicity could be a major side effect with these agents. The enhanced understanding of the pathophysiology of myeloma, intracellular pathways, soluble mediators and interactions with microenvironment has thrown up multiple potential targets in the microenvironment. This has led to creation of a pipeline of prospective agents. It still remains a major task to simplify the algorithm of studying these drugs in patients.

Fig 9: Interactions of plasma cell with the microenvironment. A detailed schematic representation of myeloma plasma cell interaction, with its cellular partners in the bone marrow microenvironment. Adapted from Podar et al Leukemia (2009) 23, 10–24

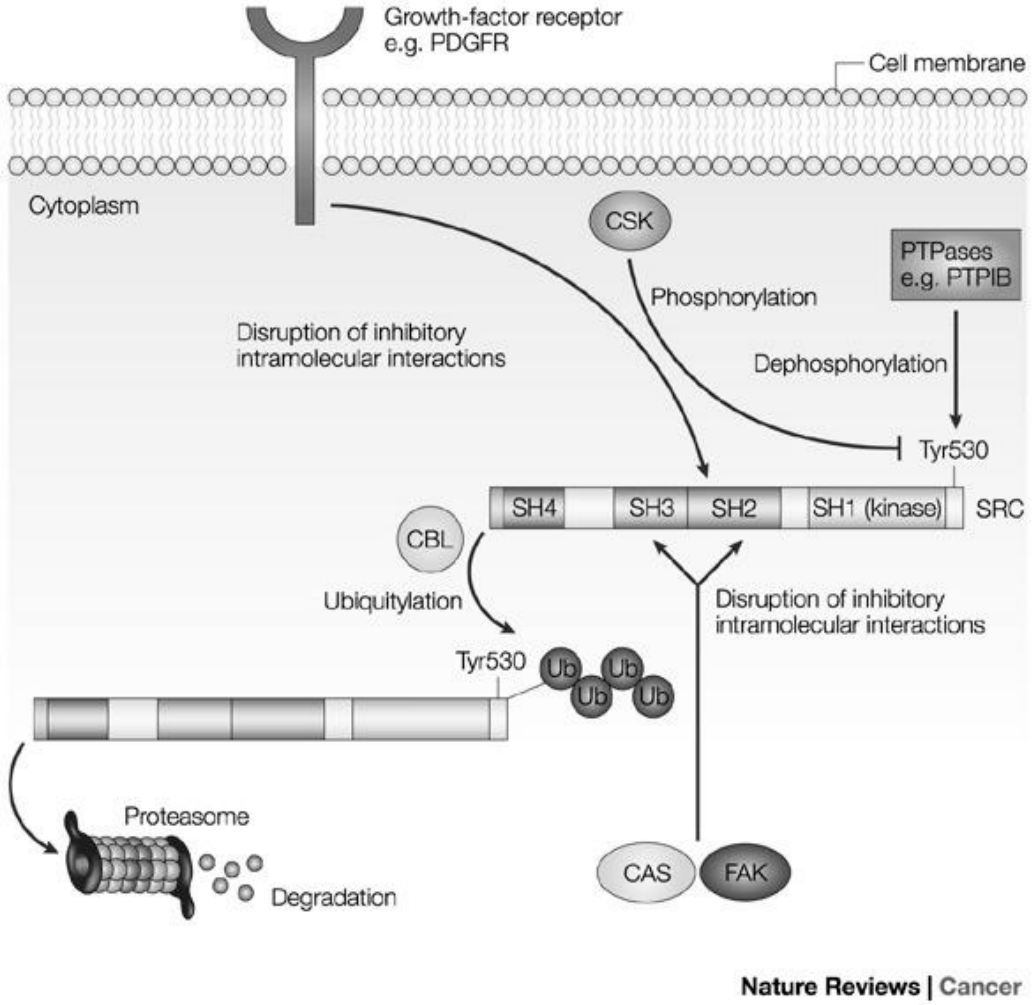


2.5 Src signalling pathways and its role in myeloma pathogenesis

c-Src was the first identified proto oncogene and has been extensively studied. Src family of membrane non-receptor protein tyrosine kinases (SFK) play a key role by transducing signals from the extracellular environment to intracellular biochemical pathways that either activate nuclear factors resulting in transcriptional responses, or target cytoplasmic components resulting in a cytoskeletal reorganisation[102]. Src participates in the maintenance of normal cell homeostasis and in a vast range of physiological functions including cell proliferation and survival, regulation of the cytoskeleton, cell shape control, and maintenance of normal intercellular contacts, cell–matrix adhesion dynamics, motility and migration. The Src family includes nine mammalian members: c-Src, Yes, Fyn, and Fgr, forming the Src A subfamily, Lck, Hck, Blk, and Lyn in the Src B subfamily, and Frk in its own subfamily [102]. c-Src is regulated in terms of both protein levels and levels of activity by a range of mechanisms. Inactivation of c-Src is controlled by c-Src tyrosine kinase (CSK), which phosphorylates a conserved tyrosine residue in the c-Src carboxy-terminal domain (Tyr530). This is reversed by phosphatases such as protein tyrosine phosphatase 1B (PTP1B), which leads to c-Src activation. When c-Src is activated it undergoes dephosphorylation of Tyr 530 and phosphorylation of 416[103]. This makes Tyr416 a useful surrogate marker for investigating Src phosphorylation in myeloma cells. In cases of overexpressed c-Src both Tyr 530 and Tyr 416 are phosphorylated. It is important to note that phosphorylation patterns in Src family members such as Lyn are different to c-Src. Activation of growth-factor receptors such as PDGFR leads to their association with the SRC homology 2 (SH2) domain, which disrupts inhibitory

intramolecular interactions thereby promoting c-Src activation. Other proteins, such as CRK-associated substrate (CAS) and FAK, bind to the c-Src SH2 and SH3 domains to promote c-Src activation by a similar mechanism (Fig. 10). c-Src when inactive is located in the perinuclear sites. Upon activation c-Src is translocated to sites of cell adhesion and interacts with the plasma membrane through its myristoylated SH4 domain.

Fig 10: Regulation of c-Src. Mechanisms involved in regulation of c-Src activity shown. CSK- c-SRC tyrosine kinase, PTB1B - protein tyrosine phosphatase 1B, CAS- CRK-associated substrate. Adapted from T J. Yeatman. Nature Reviews Cancer 4, 470-480 (June 2004)



2.5.1 SFK activation and integrin signalling in myeloma cells

Src family kinases are activated downstream of integrin signalling upon integrin engagement to ligands leading to integrin clustering. The interaction between c-Src SH3 domain and β3 integrin cytoplasmic domain is direct and other SFK bind more generally to β1, β2 and β3 integrins[104]. Formation of focal adhesions and adherens junctions are both regulated by c-Src. Focal adhesions form at the sites where integrins link the actin cytoskeleton to ECM proteins. In addition to their role as cell–matrix attachment structures, providing the structural and mechanical properties necessary for adhesion, and participate in cell-signalling processes that regulate proliferation and gene transcription. Numerous cytoskeletal proteins are recruited to focal adhesions to mediate cell migration. They are associated with cytoskeletal stress fibres, composed of actin and myosin, which control the shape and ultimately, the motility of the cell. In the context of myeloma, Src is phosphorylated in primary myeloma cells and human myeloma cell lines MM1S, MM1R, RPMI8226 and OPM1[105]. Integrin clustering results in cytoskeleton organisation followed by phosphorylation of pp60 Src and focal adhesion kinase. Src kinases also transduce signals downstream of the proteoglycan CD44. When myeloma cells adhere through CD44 variants on hyaluronan the cytoskeleton binding is intrinsically regulated by phosphorylation of Ser325. The cytoplasmic tail (Ser325) is constitutively associated with Src family protein kinases (SFK) like Lck, Fyn, and Lyn. Binding of hyaluronic acid to CD44 leads to cytoskeletal reorganization. Additionally, the Src family kinase Fyn may activate guanine exchange factor Vav1 that activates the Rho-family GTPase Rac1 and/or the PI3K/Akt/Bad pathway[106]. Levels of c-Src are higher in relapsed myeloma in

comparison with newly diagnosed patients and those with MGUS [105]. Higher levels of c-Src protein available will therefore play a key role in integrin or CD44 mediated resistance along with downstream activation of FAK and MAPK pathways.

2.5.2 Role of SFK in IL-6 signalling pathway

IL-6 is a pleiotropic cytokine, which enhances myeloma proliferation both *in vitro* and *in vivo*. High levels of IL-6 in the serum are shown to induce resistance to bortezomib therapy. IL-6 signalling is mediated by Src family kinases. Only a proportion of CD138 + plasma cells express CD45, a protein tyrosine phosphatase. Kawano et al showed that CD45 expression along with SFK activation is a prerequisite for IL-6 dependent proliferation in myeloma cell lines. CD45 neg myeloma cells highly express IL-6 receptor alpha chain (IL-6Rα). IL-6Rα and IGF-I receptors exist on plasma membrane in close proximity, facilitating efficient assembly of two receptors in response to IL-6. The synergistic effects of IL-6Rα on IGF-I receptor-mediated signals provide a novel insight into a Jak-independent IL-6 signalling mechanism of receptor cross talk in human myeloma cells [107]. Furthermore, IL-6 dependent myeloma cell line proliferation was shown to be effectively inhibited by an antisense oligonucleotide to Src kinase in vitro [108]. Another group has shown the Src family kinase Hck is associated with the IL-6R β-chain, gp130, and IL-6 signalling induces Hck activation making Hck a potential intracellular target for blocking IL-6 signalling. Peptides derived from the gp130, inhibited growth of IL-6 dependent cell lines by interfering with the association of Hck[109]. Lyn overexpressing myeloma cell lines had higher proliferative rate with IL-6 and

the enhanced activation of the phosphatidylinositol (PI) 3-kinase and Akt. The IL-6-induced phosphorylation of STAT3 and ERK1/2 was not up-regulated in the Lyn-over expressed cells, indicating that the Lyn-PI 3-kinase-Akt pathway is independent of these pathways[110]. These data confirm that Src is a potential druggable target in patients with high IL-6 levels driving the proliferation and survival of myeloma cells.

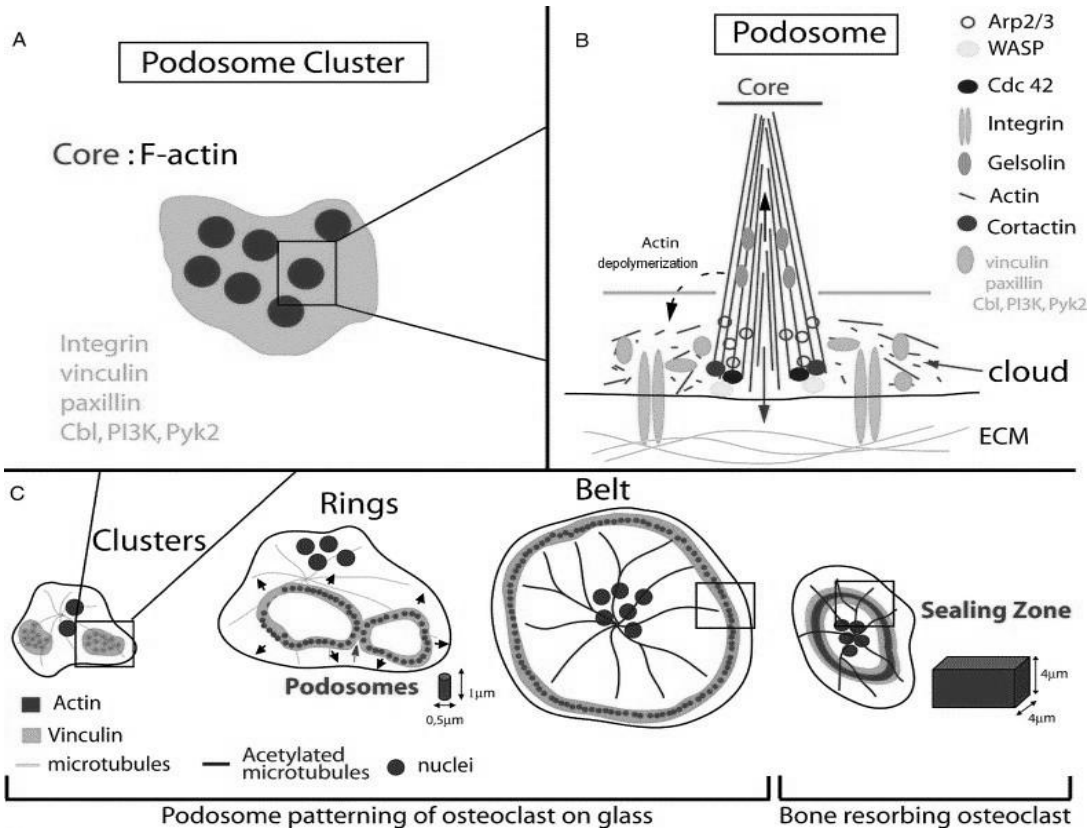
2.5.3 c-Src and bone resorbing activity of osteoclast

Src family kinases also play an important role in the motility and function of osteoclasts. Bone resorption function of osteoclasts is dependent on the integrity of the actin cytoskeleton. Depending on the substratum upon which the osteoclasts are spread, there are two different configuration of actin known as podosomes and the sealing zone (continuous ring of actin filaments along the cell periphery that arises from fusion of clustered podosomes). Formation of podosomes is a key event and is characterized by an F-actin conical core concentrating actin regulatory proteins such as WASP, Arp2/3, cortactin, and gelsolin (Fig 11). The actin core is surrounded by adhesion molecules such as integrins, adaptors (paxillin, SH3P2, Cbl, and others), kinases (c-Src, Pyk2), small GTPases (Rho and Rac), and endocytic regulators [111]. Osteoclasts are dynamic and motile cells and if plated on a suitable substratum such as bone the actin cytoskeleton switches from the migrating podosome phenotype to sealing zone found in the bone resorbing phenotype. The sealing zone consists of a large ring of actin about 4 μm wide and 4 μm high, in contrast to 1 μm in height and 0.5 μm diameter for an individual podosomes. During resorption, the sealing zone is centered at the

apical membrane of polarised osteoclasts [112]. Moreover, in resorbing osteoclasts showing sealing zone organisation, phosphorylated c-Src was mainly found in the center of the ring, in a membrane-specific region called the ruffled border. Src Tyrosine phosphorylation regulates actin polymerisation and the activity of proteins surrounding the podosome core. Tyrosine kinase activity of c-Src is also implicated in the formation of a $\alpha_v\beta_3$ integrin, Pyk2, c-Src, and Cbl complex which is regulates the resorption activity of osteoclasts. The deletion of the gene encoding the nonreceptor tyrosine kinase c-Src produces an osteopetrotic skeletal phenotype. Src–/– osteoclasts exhibit reduced motility and abnormal organization of the apical secretory domain (the ruffled border) and attachment-related cytoskeletal elements that are necessary for bone resorption. c-Src$^{-/-}$ mice are viable and fertile and do not immediately exhibit obvious phenotypes but develop osteopetrosis. Osteoclast differentiation was not affected in c-Src$^{-/-}$ osteoclasts but were unable to resorb bone in vitro. Under such conditions, the when Src$^{-/-}$ osteoclasts were cultured on cover slips, they exhibit focal adhesions but no podosomes and have a migration defect and a delayed ability to reorganise their cytoskeleton after detachment and spreading[113]. Formation of podosomes could be rescued by expression of a kinase-dead version of c-Src, highlighting the importance of the adaptor function of Src rather that its kinase activity. Furthermore, the other members of the Src family cannot compensate the absence of c-Src in the specific context of the osteoclast. Myeloma patients present with lytic lesions and high serum calcium levels which are hallmarks of osteoclast activation. Bisphosphonates down regulate osteoclast activation and are shown to be effective in myeloma patients. Targeting c-Src or other

proteins in the podosome core such as WASp should be explored in these patients [114,115]. Targeting Src family kinases with a small molecule has potential to induce plasma cell death by altering the response to IL-6, reduce adhesion of plasma cells to ECM proteins in the bone marrow and modify the microenvironment by inhibiting osteoclast mediated bone resorption.

Fig 11: Podosome structure and formation of podosomes and sealing zones in osteoclast. (A) Cluster of podosomes with core formed of F- actin. (B) Detailed structure of a podosome. Arp2/3 – actin related protein complex, WASP – Wiskott Aldrich syndrome protein, cdc42 – GTP binding protein. (C) Formation of podosome cluster, rings, belt and sealing zones on different substrates. Adapted from Jurdic et al Eur J of Cell Biol. Vol 85(3-4) Apr 2006, pg 195-202



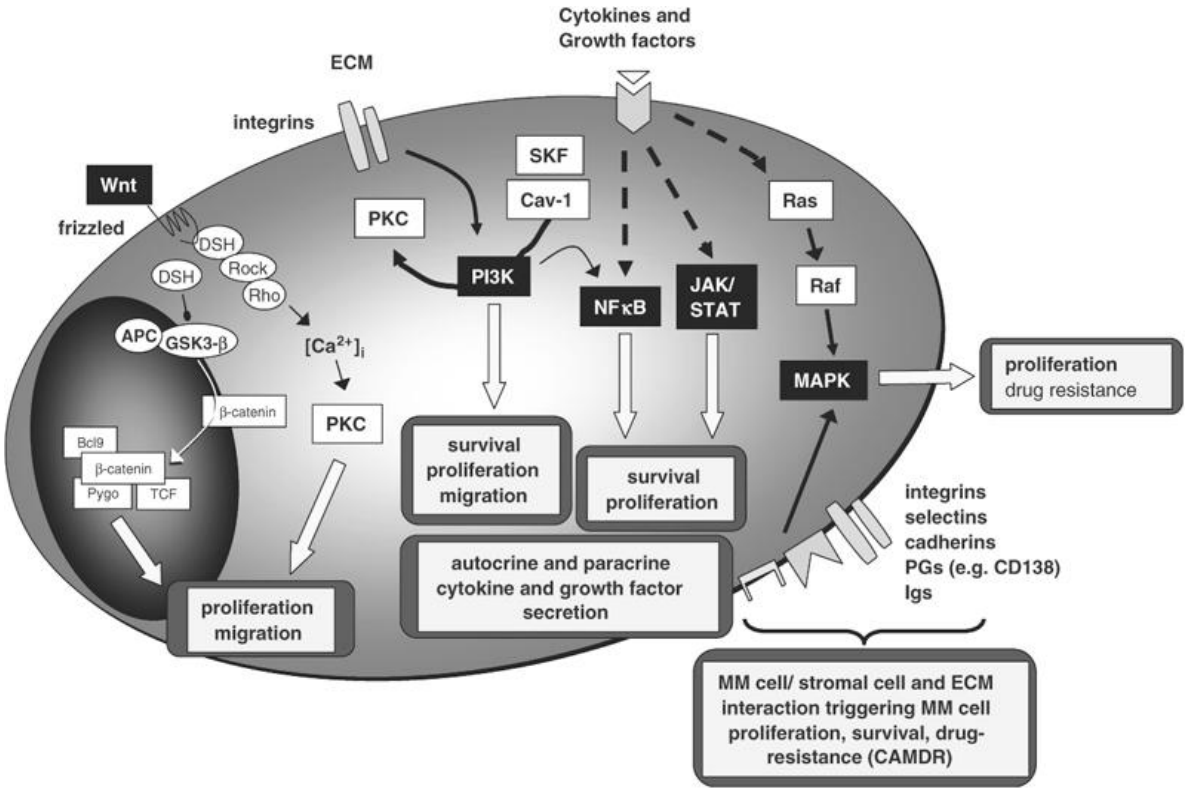
2.5.4 Kinase inhibition and its role in myeloma therapy

The success of Imatinib in chronic myeloid leukaemia (CML) has generated a huge interest in developing kinase targets for haematologic malignancies. Increasingly pharmaceutical companies are investing huge sums in identifying and blocking targets with small molecules. Myeloma therapy is going through the phase of identifying potential druggable targets which includes kinase domains either in the plasma cells or in the microenvironment. Some of the kinases in the propagation of myeloma cells have been established; vascular endothelial-derived growth factor receptor (VEGFR) kinase, insulin-like growth factor 1 receptor, Janus kinase, AKT, protein kinase C, Cdk4/6, IκB kinase β, aurora kinases and fibroblast growth factor receptor 3 (*FGFR3*). Over expression of *FGFR3* occur in a subset of multiple myeloma patients with t (4; 14) translocation. The other potential advantages of kinase therapies in myeloma are that, they can potentially be combined with manageable toxicities with the major scaffold agents such as bortezomib, dexamethasone and IMiDs[116]. In relapsing myeloma patients with poor performance status and limited suitability for high dose therapy, targeted agents such as kinase inhibitors may be more clinically relevant.

Tiedemann et al through high-throughput screening for kinase mutations in primary myeloma cell and cell lines did not detect any mutations in 31 kinases analysed except, FGFR3 [117]. The same group used a high-throughput systematic RNA interference (RNAi) approach to identify kinases that regulate myeloma cell proliferation or survival. KMS11 and INA6 human multiple myeloma cells were transfected separately with synthetic short interfering RNAs (siRNA) targeting each of the 639 known and putative kinases. JJN3

myeloma cells were used in secondary validation screens. These cell lines were chosen for their genetic heterogeneity and are representative of t (4;14) (KMS11), t(14;16) (KMS11 and JJN3), and t(11;14) (INA6) myeloma variants. Kinases targeted by 1 or more siRNAs lethal or inhibitory to KMS11 or INA6 during primary screening were implicated as candidate survival kinases. 88 kinases (14%) were identified as candidate survival kinases in KMS11 cells and 59 (9%) were identified in INA6 cells with only 4% in both cell lines reflecting the heterogeneity. G protein–coupled receptor kinase, GRK6 was identified through this process making it a candidate for drug target in myeloma[118]. Significant myeloma cell cytotoxicity is seen with inhibitors *in vitro* and with some them *in vivo*. But clinical evaluations have not been carried out particularly with concerns of toxicity with many of these agents. Potentially some of these agents will be used in combination with licensed anti myeloma drugs [119,120]. An example is AKT inhibitor Perifosine which inhibits constitutive and cytokine (IL-6, IGF-1) induced PI-3K/Akt activation in MM cells and, therefore, induces MM cell death even when the MM cells are adherent to stromal cells. Perifosine is now being trialled in combination with bortezomib and lenalidomide. FGFR3 kinase, aurora kinase, p38/MAPK, JAK1/2, protein kinase C and cyclin-dependent kinase inhibitors have been extensively evaluated in pre clinical setting (Fig 12).

Fig 12: Signalling pathways in a neoplastic plasma cell. Intracellular pathways involved in regulating key cellular processes within a myeloma plasma cell. Adapted from Podar et al Leukemia (2009) 23, 10–24



2.6 Myeloma bone disease

Bone resorption and subsequently bone destruction in the form of lytic lesions and fractures is a characteristic feature of myeloma. Although not all patients present with bone disease they could potentially develop it during the course of the disease. Abnormalities in skeletal radiography are observed in up to 90% of patients with myeloma during the course of their disease[121]. In a population-based retrospective cohort study, 16 times more fractures were observed than expected in the year before diagnosis, mostly pathologic fractures of the vertebrae and ribs. Patients with a pathological fracture had poor survival [122]. Bone disease may be limited to radiolucencies on X-rays but not infrequently patients develop pathological fractures requiring surgical pinning and radiotherapy. Bone pain significantly limits mobility and underpins the quality of life of these patients. Imbalance between bone resorption caused by osteoclastic activation with decreased osteoblastogenesis primarily leads to these clinical manifestations. Bone histomorphometric studies clearly demonstrate increased osteoclast numbers and activation in both early and overt myeloma patients [123] together with reduction in osteoblast numbers over the course of the disease. Osteoclasts are a key cellular partner in the bone marrow microenvironment of myeloma patients and enhance survival and proliferation of both autologous and allogeneic primary myeloma cells[49].

2.6.1 Osteoclast activation in myeloma

Uncoupling of osteoblast and osteoclast activity in the bone of MM patients is primarily due to activation of the osteoclast and secretion of soluble factors by plasma cells and stromal cells (Fig 11). Osteoclasts are activated by secretion of cytokines, the receptor activator of nuclear factor (NF)-κB (RANK) ligand (RANKL), the chemokines macrophage inflammatory protein (MIP)-1α. A balanced RANKL/osteoprotegerin (OPG) ratio is essential for normal bone turnover. Myeloma cells lead to an imbalance in the RANKL – OPG system in the tumour microenvironment. RANKL has been characterized as the key mediator of osteoclast differentiation and activation. RANKL is a member of the tumour necrosis factor super family and is produced mainly by osteoblastic lineage cells and stromal cells. The cellular receptor for RANKL, RANK, is expressed on the surface of osteoclast precursors and mature osteoclasts. RANKL induces differentiation, formation, fusion, and survival of pre osteoclasts. Moreover, it has direct effects on mature osteoclasts, causing actin ring formation and cytoskeletal rearrangements that precede bone resorption, and activating mature osteoclasts to resorb bone. OPG acts as a decoy receptor antagonist for RANKL. It is secreted mainly by osteoblastic lineage and stromal cells[124]. MIP-1α is chemotactic for osteoclast precursors, and promotes osteoclast formation in bone marrow cultures. Both MIP-1α and MIP-1β are produced and secreted by myeloma cells. MIP-1α and MIP-1β enhance RANKL expression in stromal cells. MIP-1α induced signalling involves activation of the PI3K /Akt and mitogen-activated protein kinase (MAPK) signalling pathways in myeloma cells, leading to increased proliferation and protection against apoptosis[125]. IL-6 and IL-11, predominantly

produced by stromal cells and IL-3 and HGF, produced by myeloma cells activate osteoclasts. Stromal derived factor alpha (SDF-1α), chemokine expressed by marrow stromal cells and myeloma cells binds to its receptor CXCR4, expressed on osteoclast precursors, inducing chemotaxis and matrix metalloproteinase (MMP)-9 activity [125]. Syndecan-1 produced by myeloma cells, along with heparanase, an enzyme involved in syndecan-1 shedding, regulates osteoclastogenesis by sequestering OPG and by concentrating factors (HGF, IL-6, IL-8, SDF-1) that promote osteoclastogenesis, angiogenesis and myeloma growth [126]. Activated osteoclasts also provide protection and enhance proliferation of neoplastic plasma cells [49]. Taken together, activation of osteoclasts in the bone marrow microenvironment of myeloma patients plays a key role in myeloma bone disease and provides several therapeutic targets, which can be exploited.

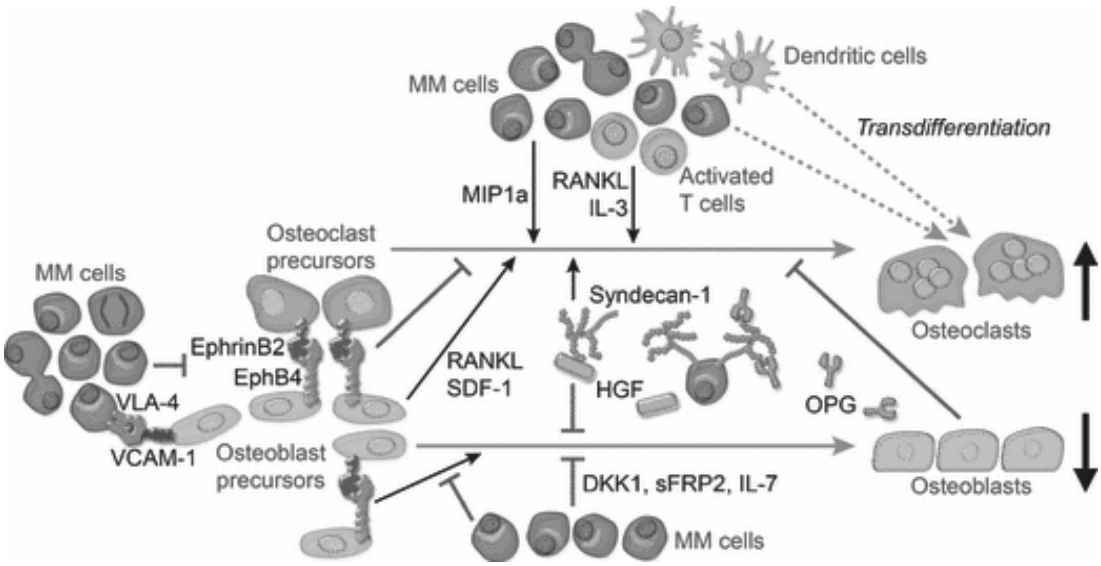
2.6.2 Osteoblastogenesis in myeloma

Suppression of osteoblastogenesis in myeloma through multiple pathways leads to suppression of bone production and promotes myeloma cell survival. Various regulatory pathways of osteoblastogenesis are altered in MM patients. Wnt pathway has been shown to be a critical signalling pathway in osteoblast differentiation. Wnt glycoproteins bind to the Wnt receptor and its co receptors low-density lipoprotein receptor-related protein (LRP) 5 /LRP6. This leads to stabilisation and cytoplasmic accumulation of β-catenin. β-catenin translocates into the nucleus and stimulates expression of osteoblastic target genes. In the absence of a *Wnt3a* (Wnt glycoprotein) signal, β-catenin is phosphorylated and degraded by the proteasome. Tian et al reported that

Dickkopf 1 (DKK1) a soluble Wnt signalling antagonist secreted by plasma cells and stromal cells inhibits osteoblastogenesis[127]. Patients with myeloma who had high levels of serum DKK1 had significant bone disease. Inhibition of DKK1 using a monoclonal antibody in vivo induced osteoblast mediated bone formation in SCID-rab mice [128]. Secreted Frizzled-related protein 2 (sFRP-2), a Wnt inhibitor is produced by RPMI8226 and U266 human myeloma cell lines and primary myeloma cells. sFRP-2 suppressed in vitro mineralization as well as alkaline phosphatase activity in osteoblasts induced by bone morphogenetic protein 2 (BMP-2). Decorin, a small leucine-rich proteoglycan that is produced by osteoblasts, inhibited the survival of myeloma cells and attenuated the stimulatory effects of osteoclasts on myeloma cells. They also demonstrated that blocking activity or expression of decorin reduced osteoblasts inhibitory effects on myeloma cell growth and survival, but over expression of decorin in mesenchymal stem cells (MSCs) lessened the ability of these cells to support myeloma cell survival[129]. Cell surface receptor EphB4 and its ligand ephrinB2 mediate bidirectional signalling between osteoblasts and osteoclasts. Osteoblasts and their precursors, mesenchymal cells, express ephrinB2 and EphB4, whereas osteoclasts mainly express ephrinB2. Forward signalling (activation of ephrin receptors by ligands) in MSCs promotes osteogenic differentiation, and reverse signalling (activation of ligands by receptors) in osteoclast precursors inhibits their differentiation into multinucleated bone-resorbing osteoclasts[130]. EphrinB2 and EphB4 are underexpressed in MSCs from patients with MM. *In vitro*, myeloma cells downregulated expression of these factors in normal mesenchymal cells and had no effect on expression of ephrinB2 in osteoclast precursors[131].

Drug therapeutic strategies against myeloma ideally should promote osteoblast metabolism as well. Cbfa1/Runx2 is a transcription factor essential for bone formation and osteoblast differentiation. Proteasomal inhibitor Bortezomib significantly increased the transcription factor Cbfa1/Runx2 activity in human osteoblast progenitors and osteoblasts *in vitro*. This correlates with increased numbers of osteoblasts in the marrow of patients treated with bortezomib[52].

Fig 13: Bone remodelling in myeloma. Mechanisms leading to disruption of bone homeostasis. Adapted from Yaccoby.S. Brit J of Haematology (2009) Vol 149 (3) **p 311-321)**



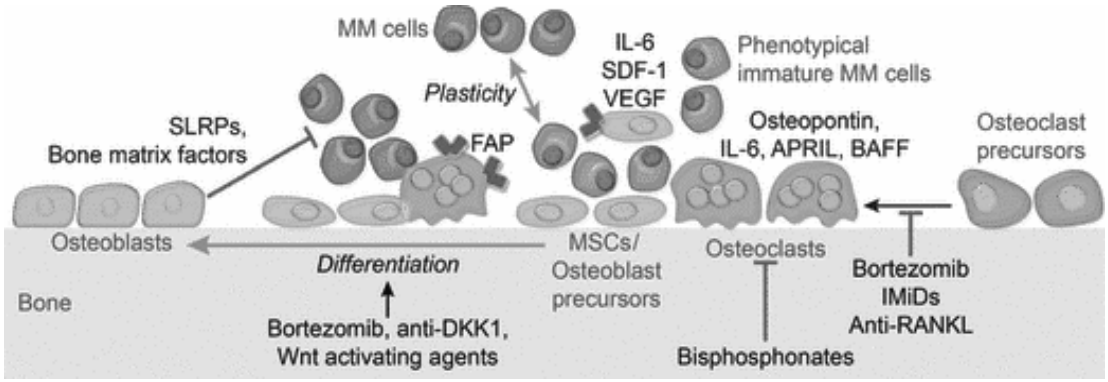
2.6.3 Therapeutics for myeloma related bone disease

Until recently, targeting osteoclast activation has been the mainstay of therapy for myeloma related bone disease. Frequently, orthopaedic intervention kyphoplasty, vertebroplasty and radiotherapy are used to improve structural integrity and alleviate pain. The aim of therapy is the reduction of skeletal-related events such as fractures, bone pain and plasmacytomas in patients with myeloma bone disease. Phase III randomised, placebo-controlled clinical trials have proven the efficacy of intravenous pamidronate or oral clodronate in the therapy of myeloma bone disease. Bisphosphonates inhibits osteoclast activation and induces osteoclast apoptosis. Zoledronic acid is superior to pamidronate in the treatment of hypercalcemia of malignancy, but a clinical efficacy comparable with that of pamidronate in myeloma in the prevention of skeletal-related events[132]. A recent Cochrane review showed bisphosphonate in comparison with placebo, demonstrated the beneficial effect on prevention of pathological vertebral fractures (RR= 0.74 (95% CI: 0.62 to 0.89), P = 0.001), and skeletal related events (SREs) (RR= 0.80 (95%CI: 0.72 to 0.89), P < 0.0001). There was no significant improvement in OS or PFS. They did not find a significant difference in efficacy between the Bisphosphonates [133]. Recently concluded myeloma IX trial showed superiority of zoledronic acid over oral sodium clodronate for OS and PFS with the overall survival independent of occurrence of SREs[134]. Osteonecrosis of the jaw (ONJ) is a rather uncommon, but potentially serious, complication of intravenous bisphosphonates, which is characterised by the presence of exposed bone in the mouth. Invasive dental procedures are a major risk factor for the development of ONJ. Zoledronic acid use was associated with a higher

incidence of ONJ in a recent meta analysis [135]. Various alternative compounds that may target MM bone disease are under investigation. Recently, Denosumab a RANKL neutralizing antibody known to be effective in osteoporosis was trialled in phase II setting in patients with relapsed or plateau-phase myeloma. Some patients who were treated with RANKL-neutralizing antibody denosumab experienced MM disease stabilisation[136]. A humanised DKK1-neutralizing antibody, BHQ880, which is currently being evaluated in clinical trials, has shown increased osteoblast numbers and bone mass and reduced osteoclast numbers in SCID-hu mice engrafted with the IL6-dependent myeloma cell line INA6[137]. Inhibition of the ubiquitin–proteasome pathway induces osteoblast differentiation by increasing expression of bone morphogenetic protein and preventing proteolytic degradation of RUNX2. Bortezomib also promotes bone formation by inhibiting DKK1 expression in osteogenic cells[52]. Long term treatment with Imatinib (Abl kinase inhibitor) in CML patients increased bone formation by inhibiting osteoclastogenesis suggesting that chronic therapy with a kinase inhibitor has the capacity to help bone remodelling[138],[139]. The effects are thought to be related to inhibition of PDGF, c-fms, and abl kinase. Dasatinib showing promising activity in CML shows multi-target effects, including inhibition of the macrophage colony stimulating factor (M-CSF) receptor, c-fms, c-Abl and c-Src. Dasatinib abrogates osteoclast formation and activity in vitro and in vivo in Sprague Dawley rats[140]. The increase in trabecular volume observed was comparable to zoledronic acid. This observation is primarily due to impaired osteoclast activation and it remains to be explored, whether similar effects would be obtained in myeloma patients with defective

osteoblastogenesis. In summary, to arrest the progression of bone disease in myeloma, the goal of therapy should focus on inhibiting osteoclast function and enhancing osteoblastogenesis (Fig 14).

Fig14. Therapeutic targets in myeloma bone disease. Existing and potential druggable targets with potential to modify myeloma bone disease. Adapted from Yaccoby.S, Brit J Haem (2009) Vol 149 (3) p 311-321)

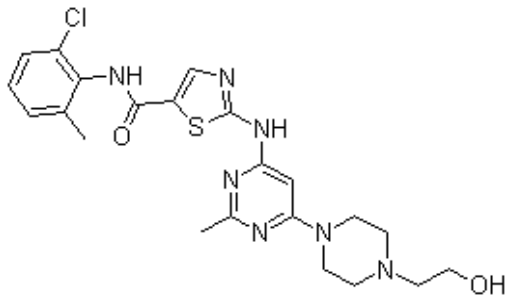


2.7 Dasatinib therapy for myeloma

Dasatinib is an oral, multitargeted tyrosine kinase inhibitor of members of five tyrosine kinase families: SRC family kinases (SRC, LCK, YES, FYN), BCR-ABL, KIT, EPHA2 and PDGFβ. It has been shown to be safe and effective in CML patients, who are resistant and intolerant to Imatinib mesylate. Dasatinib is the analog of parent compound 2-aminothiazole discovered as a novel Src family kinase inhibitor. Molecular modelling was used to construct a putative binding model for Lck inhibition by this class of compounds[141]. Dasatinib has a chemical name, N-(2-chloro-6-methylphenyl)-2-[[6-[4-(2-hydroxyethyl)-1-piperazinyl]-2-methyl-4-pyrimidinyl] amino]-5-thiazolecarboxamide, monohydrate according to the IUPAC nomenclature (Fig 15). *In vitro* studies have demonstrated that Dasatinib is 150 fold more potent than Imatinib in inhibiting bcr-abl and in addition inhibits Src kinase in nanomolar concentrations. Safety and efficacy of Dasatinib has also been demonstrated in several Phase II and Phase III trials in CML patients in chronic, accelerated and blast crisis phase of disease[142]. Dasatinib is also being studied in Philadelphia positive ALL, prostate cancer and melanoma. Up to one third of patients in chronic phase CML develop resistance/sub optimal response to Imatinib therapy. There are also patients who are intolerant to Imatinib. In a phase III randomised study, 519 patients with newly diagnosed chronic-phase CML were randomly assigned to receive dasatinib at a dose of 100 mg once daily (259 patients) or imatinib at a dose of 400 mg once daily (260 patients). After a minimum follow-up of 12 months, complete cytogenetic response and molecular response was higher with Dasatinib than with Imatinib (77% vs. 66%, P=0.007), and (46% vs. 28%, P<0.0001) respectively. The main side

effect restricted to the dasatinib group was pleural effusion, reported only in 26 patients (10%) and no grade 3-4 events. The tolerability to Dasatinib has significantly improved since the reduction dose from 70mg BD to 100mg OD [143]. Direct comparison with Imatinib mesylate in a randomised fashion as first line therapy for chronic myeloid leukaemia patients in chronic phase shows superior complete cytogenetic response, which is a surrogate marker of long term remission [144]

Fig 15: Chemical Structure of Dasatinib. Adapted from Simard et al Nature Chemical Biology 5, 394-396 (2009)



2.7.1 Potential targeting of microenvironment by Dasatinib: Inhibition of angiogenesis

The most important molecule identified to date, that controls angiogenesis is vascular endothelial growth factor A (VEGF A) which belong to a family of potent angiogenic regulators. VEGF A binds to VEGFR1 (FLT1) or VEGFR2 (KDR) which have tyrosine kinase activity. Notch receptor and its ligand DLL4 and PDGFRβ and its ligand PDGF which are highly expressed in pericytes are also implicated both in physiological and tumoral angiogenesis [145]. Dasatinib demonstrates antiproliferative activity in human umbilical vein endothelial cells (HUVEC) treated with VEGF or basic fibroblast growth factor (bFGF). IC50 values of 42 nM and 248 nM were obtained under conditions with VEGF-stimulated and bFGF-stimulated growth, respectively[140]. Additionally, Dasatinib attenuates VEGF and bFGF stimulated HUVEC endothelial cell migration in vitro (IC50<5 nM)[140]. This demonstrates that Dasatinib has an effect on neovascularisation, maturation and migration of endothelial cells. Vacca et al took plasma cells and endothelial cells form myeloma patients (MMEC). They studied PDGFRβ and pp60c-Src which are constitutively activated in both cells. They demonstrated PDGF-BB/PDGFRβ kinase-axis promoted MM tumour growth and vessel-sprouting by activating ERK1/2, AKT, upon treatment with VEGF, released by MMEC. They have also shown using siRNA experiments they have validated that pp60Src is key signalling effector molecule in VEGF loop induced angiogenesis in MMEC. Blocking PDGFRβ and pp60c-Src in vitro and in vivo using Dasatinib , they have shown reduction in angiogenesis and tumour growth [105]. In a recent Phase I/II study of the Src inhibitor Dasatinib in combination with erlotinib in

34 advanced non-small-cell lung cancer patients, significant reduction in plasma VEGF and bFGF were observed. The reductions in VEGF correlated with disease control[146]. As described in section 2.1.2 of this thesis, angiogenesis plays an important role in the progression of myeloma. But angiogenesis as potential therapeutic target still remains unclear because of modest responses to bevacizumab (VEGF inhibitor) and modest reduction of angiogenesis in patients responding to thalidomide. It still remains to be demonstrated that angiogenesis is an important facet in myeloma, particularly as extramedullary plasmacytomas develop in relatively avascular areas and angiogenesis in marrow of relapsed patients is thought to be a reflection of tumour load.

2.7.2 Potential targeting of microenvironment by Dasatinib: Inhibition of bone resorption

Osteoclast mediated bone resorption is a complex process, intricately involving several steps coordinated by humoral factors, activation of signalling pathways in the osteoclast, engagement of podosomes on an appropriate substrate followed by release lytic enzymes. Hormones such as testosterone, oestrogen, parathyroid hormone (PTH)-related peptide (PTHrP), Parathyroid hormone along with TNF-α, IL-1β, $1,25(OH)_2$ vitamin D_3 regulate bone resorption through RANKL expression. Five signalling pathways mediated by protein kinases are induced during osteoclast activation; inhibitor of NF-κB kinase (IKK), c-Jun N-terminal kinase (JNK), p38, extracellular signal-regulated kinase (ERK) and Src pathways. Src protein, required for osteoclast activation, binds TRAF6 and allows RANK-mediated signalling to proceed through the lipid kinase (PI (3) K) and the serine/threonine protein

kinase AKT. Once osteoclast forms a sealing zone on the substrate, acidification of this compartment by secretion of protons leads to the activation of tatrate resistant acid phosphatase and cathepsin K, the two main enzymes responsible for the degradation of bone mineral and collagen matrix[147]. In preclinical models, Dasatinib has shown potent Src inhibitory activity and c-Src phosphorylation is downregulated at a serum concentration of 42nM[140]. Dasatinib dose-dependently inhibits PTH stimulated release of calcium into the medium by foetal rat long bones in vitro with an apparent IC50 of 2nM. At 5nM Dasatinib completely blocked PTH-stimulated bone resorption in vitro. Moreover, Dasatinib blocked the normalisation of plasma calcium in the thyro-parathyroidectomised rat model after PTH infusion [140]. In a phase II study of chemotherapy naive 40 castration resistant prostate cancer patients, Dasatinib induced reduction in urinary N-telopeptide levels and bone specific alkaline phosphatase in at least half of them, confirming reduction in bone resorption[148]. There is conflicting evidence as to whether Dasatinib suppresses osteoblast proliferation and differentiation by down regulating c-Src and c-Abl in the osteoblasts [149,150]. In summary, Dasatinib demonstrates potent inhibition of bone resorption in preclinical models and patients without significant toxicity making it an exciting drug to use in myeloma patients who have activated osteoclast mediated bone resorption.

2.7.3 Dasatinib as a therapeutic agent in myeloma

Dasatinib significantly delayed MM tumour growth and angiogenesis *in vitro* and *in vivo* in SCID mouse models. Dasatinib also exhibited a synergistic cytotoxicity with chemotherapy drugs and bortezomib [105]. However, the efficacy of Dasatinib as a single agent in MM patients was not significant. A

single-arm, phase II, open-label study of Dasatinib in 21 myeloma patients with relapsed or plateau phase was performed. Dasatinib was administered continuously at an oral dose of 70 mg twice daily, on Days 1 through 28, with cycles repeated every 28 days. After the enrolment of 9 patients and no observed responses, the protocol was amended to allow dose escalation of Dasatinib in patients with stable disease after 8 weeks on therapy to 100 mg twice daily. The overall median duration of therapy was 51 days. The overall response rate was low at 4.8%. Significant toxicity was observed with Grade 3 or more haematologic and non haematologic toxicity observed in up to one third of patients[151]. Hence, Dasatinib as a single agent lack potential as a therapy against MM despite the observed significant efficacy of this drug at blocking the activity of BM cells that may support MM viability in preclinical and some clinical studies. Some *in vitro* studies suggest synergy for direct MM killing of Dasatinib with other drugs. Deng et al have also shown in human myeloma cell lines there is differential sensitivity to Dasatinib and synergistic activity with steroids, bortezomib and lenalidomide[152]. In myeloma cell lines, Dasatinib activated caspase-8 and caspase-12, and sensitised myeloma cells to agents that activate caspase-9, such as dexamethasone and bortezomib. They were also able to overcome IL-6 mediated and stroma mediated resistance in myeloma cells [152]. Imatinib inhibits bcr-abl, c-kit, and platelet-derived growth factor beta (PDGFRβ). Imatinib was trialled as monotherapy in Phase I/II setting involving 21 relapsed/refractory myeloma patients, no responses were observed[153]. This observation was remarkable as one third of myeloma patients express c-kit on the plasma cells. **I hypothesised; inhibiting SFK activation with Dasatinib would predominantly block the**

activity of protective BM microenvironment cells, may inhibit adhesion of MM cells to BM cells and extracellular matrix and impair osteoclast function. I propose Dasatinib would therefore sensitise the tumour clone and increase susceptibility to dexamethasone.

PATIENTS, MATERIALS AND METHODS

3. Patients, Materials and methods

3.1 Patients

Myeloma and MGUS patients as part of routine clinical testing consented to bone marrow (BM) and peripheral blood (PB) research samples to be taken. Patients gave written informed consent on the LREC protocol No 02-044. The Kings College Hospital Research Ethics committee approved the LREC protocol. The primary samples once obtained from the clinical facility were anonymised and if the sample was not used immediately for laboratory experiments, the sample was banked according to protocol in the Human tissue authority approved local tissue bank. The clinical information on the myeloma and MGUS patients were procured from the data available in the tissue bank database.

3.2 Obtaining Osteoclasts from Primary Samples

BM and PB were diluted with Hank's balanced salt solution, Sigma in a ratio of 1:1. Bone marrow particles were sheared using 18G needle. Density gradient separation (Histopaque, Sigma) was used to isolate BM mononuclear cells and PB mononuclear cells from patient samples. Cells were counted using a Haemocytometer. In case of bone marrow mononuclear cells, cells were plated overnight at 3 X 10^5cells/ ml in DMEM + 10% FCS and 5ng/ml of CSF-1. The next day bone marrow cells in the supernatant (adherent fibroblasts in the flask) were taken and plate din 12 or 24 well plates. They were plated at a density of 1.5X10^5 cells per well in a 96 well plate or 5 x10^5 cells per well in 24 well plate in DMEM +10%FCS with Human CSF-1 (Peprotech, UK) 25ng/ml and 50ng/ml of OPG – Ligand (Peprotech, UK).

Peripheral blood mononuclear cells were plate at a density of 3 x 10^6 cells/ ml in a 24 well plate and left to adhere for 3 hours. After 3 hours the supernatant with non adherent cells were discarded and the wells replenished with DMEM + 10% FCS fortified with CSF-1 (Peprotech, UK) 25ng/ml and OPG- Ligand (Peprotech, UK) at 50ng/ml. Cultures were aspirated and fed with fresh conditioning medium every 72 hours. Osteoclasts were fully grown by Day 17.

3.3 Cell lines and Reagents

MM1S and MM1R human myeloma cell lines were gifted by Dr.Tai (DFCI Boston, USA) and were cultured in RPMI1640 (Sigma) with L-Glutamine and 10% FBS (Sigma), Penicillin G (500U/ml) and streptomycin (50mg/ml) (sigma). ARH77, RPMI 8226 and U266 obtained from the American Type culture collection (LGC Promochem, Middlesex, UK) were maintained in RPMI Dutch modification (Sigma) and 10% FBS (Sigma) except for U266 -15%, Penicillin G (500U/ml) and streptomycin (50mg/ml) (Sigma), L-glutamine 200um (Sigma) and 1%Sodium Pyruvate (Sigma). 5T33 and 5T33 GFP cell lines were obtained from Dr. Sirac Dilber, Karolinska Institute, Sweden and maintained in Minimal Essential medium Eagle (Sigma) 10% FBS (Sigma), Penicillin G (500U/ml) and streptomycin (50mg/ml) (Sigma), L-glutamine 2mM/L (Sigma) and 1% Sodium Pyruvate (sigma). All cell lines were cultured at 37^0C in 5%CO_2.

3.4 Methyl thiazolyl tetrazolium (MTT) colorimetric assay

Using a growth curve was adequate density for plating in proliferation experiments for MM1R, RPMI8226, ARH 77 and U266 cell lines was 2X10^5 cells/ml, whereas MM1S, 5T33 and 5T33 GFP pos proliferated well without a plateau at a density of 4X10^5 cells/ml over a 5 day period. Cell lines were plated at a density of 2X10^5 cells/ml in a 96 well plate in quadruplicate, either untreated or with drugs. On the days of assessment 15ul of MTT (5mg/ml) solution (Sigma) was added to the wells. Plate was incubated for 3 hours at 37^0C in an incubator at 5%CO_2. The plate was then spun down at 625Gy and aspirated. Stop solution (0.1N Hcl in Isopropanol) was added and plate read at 570nm wavelength using a plate reader.

3.5 Flow cytometry for apoptosis measurement and cell cycle analysis

Cell lines were plated at a density of 2X10^5 cells/ ml in 12 well plates. On the days of analysis, cells were spun down at 450Gy. Cells were resuspended in Annexin V binding buffer with Annexin V APC conjugated (BD Pharmingen, UK) at a working concentration of 10µl/10^6 cells for 15 minutes in the dark. Centrifuge at 300Gy. Cell pellet was resuspended in Annexin V binding buffer 200uL. 5µl / tube of 7-AAD was added and the cells were incubate din dark for 15 minutes prior to analysis. For cell cycle analysis, eGFP expressing MM cells were harvested and fixed in BD Cytofix/ Cytoperm™Fixation/ Permeabilization. Samples were washed twice (5 min, 450 × *g*) in PBS and resuspended in a PBS solution containing propidium iodide (50 µg/ml) (sigma) and RNase (75 U/ml) (sigma) for 30 min at room temperature in the dark. Fluorescence emitted per cell unit was measured by flow cytometry using

appropriate filters using a BD FACSCanto II flow cytometer (BD Biosciences). All the experiments were repeated three times.

3.6 Adhesion assays

Adhesion on Integrin Ligands: 96 well plates were coated overnight with Polylysine (Sigma) 10ug/ml and Fibronectin (Sigma) 10ug/ml. Plates were washed with Phosphate buffered saline (Sigma) and 5% BSA was added for 30 minutes to prevent non specific adhesion. Cell lines were seeded in 96 well plate in RPMI at density 1X10^5 cells per well. They were left either untreated or treated with drugs. The cells were left to adhere for 30 minutes. Supernatant was aspirated and wells washed with RPMI 1640 once prior to adding 25ul MTT solution. MTT assay was performed.

Adhesion on Osteoclasts: Myeloma cell line ARH77 was labelled with CFSE (Vybrant CFDA SE Tracer kit, Invitrogen, UK) as per protocol. In the first experiment CFSE labelled ARH77 cells were added at a density of 1x10^5 to fully grown Osteoclasts in DMEM only with or without MgEGTA for 30 min, to activate integrins and thereby enhance adhesion. They were left either untreated or treated with Dasatinib, Dexamethasone or the combination. In the second experiment prior to plating the CFSE labelled ARH77 cells for 30minutes, the plasma cells or osteoclasts were pre-treated for 60 minutes with drugs. After aspirating the supernatant the plates were read using Tecan Fluorescent plate reader.

3.7 Western blot

Cell lines were plated at a concentration of 4x 10^5 cells/ml in 24 well plates precoated with 10ug/ml of Poly-L-lysine (Sigma) or 10ug/ml of fibronectin (Sigma). After adhesion for 30 minutes the supernatant was aspirated and wells washed once with media. Then 100ul of Laemmli buffer 1X was added to the wells. Laemmli buffer 4X was made using 0.8 g SDS stock (Sigma), 4 ml 100% glycerol (Sigma), 0.01% bromophenol blue. Final Concentration is .02% (Sigma), 1 ml ß-mercaptoethanol (electrophoresis grade) (Sigma) and 2.8 ml water. This was further diluted in water 1in 4 parts to make up 1x buffer. Lysates were sheared using 1ml syringe and needle and heated to 100°C for 5 minutes and stored down at -20°C until electrophoresed. 10% SDS PAGE resolving gel and 4% SDS PAGE stacking gel were prepared. 10ul of Lysates were run with 5ul of Dual precision plus standards (Biorad, UK) in Biorad Power Pac Basic system. Once the standards had fully resolved the gel was transferred onto a Nitrocellulose membrane (Amersham, UK) over 4 hours. The membrane was stained with Ponceau S solution (Sigma) to check adequate transfer. The membranes were blocked with BSA 5% in TBS-T blotted with primary antibodies pSrc, c-Abl activity kit, C-Abl, AKT, pAKT, ERK, pERK (Cell signalling, UK) and c- Src clone GD11 (Upstate Biotech) & B-actin (Sigma) overnight at 4°C. HRP linked secondary antibodies were added to these membranes for an hour. ECL or ECL Plus Chemiluminescence kit (Amersham, UK) was used to detect presence of the bands. Konica – Minolta SRX-101-A photo developer was used to develop X-ray films obtained from Fujifilm. The films were scanned in HP scanner and edited using Adobe photoshop CS3.0 software.

3.8 Immunofluoroscence

Once osteoclasts were fully formed they were left untreated for a further 72 hours or treated with drugs. 100ul 4% PFA solution in PBS was added to fix the cells until Immunostaining was performed. Plated were washes with PBS the Osteoclasts on the cover slips were treated with Triton 0.05% (Sigma, UK) to enable intracellular staining. Cover slips were removed from the plate and blocked with BSA 5% in PBS for 45 minutes. Primary antibodies Beta 3 integrin (serotec) and Vinculin (Sigma) were added at a concentration of 1:100 for 60 minutes. Secondary Antibodies and Phalloidin Alexafluor 568 were added for 1 hour. Cover slips were washed and inverted onto a slide with a drop of Vectashield mounting medium with DAPI (Vector labs, UK). The slides were imaged using a Fluorescent microscope (Leica).

3.9 Statistical Analysis

Calcusyn software version 2.0 (Biosoft, Ferguson, MO, USA) was used to analyse whether drug effects were synergistic or additive. The software performs multiple drug dose-effect calculations using the Median Effect methods described by T-C Chou and P. Talalay. The method for drug combination is based on the median-effect equation, derived from the mass-action law principle, which is the unified theory that provides the common link between single entity and multiple entities, and first order and higher order dynamics. A Combination Index (CI) value of <1.0 indicates synergy and >1.0 is additive. Significance of the drug effects on Proliferation and adhesion of myeloma cell lines were analysed using paired samples T-test

3.10 Generation of eGFP expressing MM cell lines

Lentiviral vector stocks were produced in 293T cells by co-transfecting the transfer vector pHR_SINcPPT-SFFV-eGFP-(SEW), the envelope plasmid pMD.G, and the packaging plasmid pCMVR8.91, as previously described [154]. 1.5 X 10^7 cells were seeded onto 150 cm^2 flasks and transfected with 10 μg DNA envelope, 30 μg DNA packaging and 40 μg DNA transfer vector by precomplexing with 0.125 μM PEI (22 kDa) for 15 minutes at room temperature in OptiMEM. After 4 hours at 37°C the medium was replaced with fresh DMEM 10% FCS and virus were harvested 48 and 72 hours post transfection. After filtering through a 0.45 μm-pore-size filter, the virus suspension was concentrated by centrifugation at 50,000 g for 2 hours at 4°C. The resulting pellet was resuspended in RPMI (Sigma, UK) and stored at –80°C until use. The desired numbers of plasma cells were plated in complete culture medium as described above and lentivirus-containing supernatant was added to the cells at an MOI of 10 and incubated for 24 hours. Medium was replaced with complete culture medium after 24 hours and cells were cultured for a further 48 hours to allow maximal expression of lentiviral vectors. eGFP expressing MM cells were resuspended at a density of 4 cells per ml in 250 μl/well in 96 well plates. After a period of 3 weeks, clones of cells in multiple wells were harvested and expanded in a 6 well plate with fresh media. Expression of GFP was confirmed by flow cytometry

3.11 Fluorimetry

eGFP-MM1S and eGFP-MM1R Cell lines were plated at a density of 4 x 10^5 cell/ml and 2 x 10^5 cell/ml, respectively in a 96 well plate in quadruplicate,

either untreated or with drugs. On the days of assessment, the fluorescence intensity (FI) per well was read at λex488nm/λem528nm using a FLx800 multidetection microplate reader (Biotek Instruments, USA). FI data were collected using Gen5 1.07 software.

3.12 Stromal cell co-cultures

HS5 human stromal cell (SC) line was obtained from American tissue culture collection. HS5 cells were cultured at 37°C in a humidified atmosphere in the presence of 5% CO_2 in DMEM supplemented with antibiotics (penicillin at 100 U/ml, streptomycin at 100 µg/ml) and 10% FBS. For cell proliferation assays of MM cell lines in co-culture, SCs were plated at a density of 0.3 x 10^5 cells per well in a 96 well plate overnight. Media was aspirated and eGFP positive MM cell lines were plated at a density of 4 x 10^5 cells/ml (eGFP-MM1S) and 2 x 10^5 cell/ml (eGFP-MM1R) on the semi-confluent SCs. On the days of assessment, plates were read in FLx800 multidetection plate reader as described above. For FACS assays, SCs were plated at a density of 10^5 cells/well overnight in a 24 well plate. GFP positive plasma cells were added at the above density with and without treatments. On the days of assessment, suspended cells (which corresponded mainly to PC) were collected and wells washed with PBS once to retrieve the remaining PC which were loosely adhered on SCs. We confirmed by flow cytometry that the remaining cells in the well were eGFP negative corresponding to SCs only.

3.13 Osteoclast co-cultures

Osteoclasts were differentiated over 3 weeks from primary BM mononuclear cells obtained from MM patients as previously described[44]. GFP positive plasma cells were layered on fully grown osteoclasts in 96 well plates at densities described above. On the days of assessment, images were obtained using a Fluorescent microscope and fluorimetry performed using a multi detection plate reader FLx800. For flow cytometry analysis, GFP positive PC were plated on fully grown osteoclasts in 24 well plates at above densities and supernatant containing PC was collected on the days of assessment. Wells were washed with PBS once to collect the remaining plasma cells. We confirmed by flow cytometry that the remaining cells in the well were eGFP negative, corresponding to osteoclasts only.

3.14 Image processing

Images of eGFP-MM cells in co-culture with osteoclasts were captured using a Nikon inverted fluorescent microscope attached to a digital camera. Images were processed in Photoshop CS3 as described before [155] to transform the fluorescent image into a binary black and white image. Firstly, the image mode of the fluorescence image was transformed from RGB to greyscale. Then, the image was enhanced using the autolevels function to make the fluorescent signal sharper and more distinguishable from the background. Using the Threshold function, the cells outline is drawn so that the cells are shown in white pixels and the background in black pixels. Images were then processed using UTHSCSA ImageTool (IT) v3.0, a free image processing software for image analysis. Using IT we transformed the enhanced images

into binary images using automatic threshold and calculated the percentage of the area occupied by white pixels corresponding to eGFP-MM cells.

3.15 Bone resorption assay

BM mononuclear cells and PB mononuclear cells from patient samples were separated using Histopaque as described in section 3.2. In case of bone marrow mononuclear cells, cells were plated overnight at 3 X 10^5 cells/ ml in DMEM + 10% FCS and 5ng/ml of CSF-1. Dentine discs (IDS ltd, Bolton, UK) were added in a sterile environment to the 96 well plates with graphite pencil marking to denote the surface opposite to the culture surface. The next day bone marrow cells in the supernatant (adherent fibroblasts in the flask) were taken and plated on dentine slices in 96 well plates. They were plated at a density of 1.5X10^5 per well in DMEM +10%FCS with Human CSF-1 (Peprotech, UK) 25ng/ml and 50ng/ml of OPG – Ligand (Peprotech, UK). In case of peripheral blood mononuclear cells, they were plate at a density of 1 x 10^6 cells/ml in a 96 well plate with dentine slices in the base of the wells and left to adhere for 3 hours. After 3 hours the supernatant with non adherent cells were discarded and the wells replenished with DMEM + 10% FCS fortified with CSF-1 (Peprotech, UK) 25ng/ml and OPG- Ligand (Peprotech, UK) at 50ng/ml. On Day 12 drugs, dexamethasone 1uM, Dasatinib 150nM, the combination of dasatinib and dexamethasone were added to the cultures. After 5 days of drug treatment the dentine slices were taken out and washed with PBS. They were treated with 50µl of 1M NH_4OH overnight to remove the adherent osteoclasts. Sonication (Soniprep MSE 150, Wolf labs, UK) was carried out for 15 seconds thrice in 1 ml PBS to further rid the dentine slices of

cell debris. The dentine slices were washed in PBS and dried. For staining, 50µl of Toluidine blue 0.1M was added to the dentine slices for 30 seconds and washed off with PBS. The dentine slices were dried before imaged under the phase contrast settings on the microscope (Zeiss microscope)

3.16 Cytokine ELISA

MIP-1α ELISA: MM1S and MM1R cells were plated at a density of 2 x 10^5 cells/ml in 96 well plates with and without drugs for 24 hours. Dasatinib 150nM, Dexamethasone 1uM and combination of Dasatinib and dexamethasone were used. The supernatants were taken after 24 hours and a MTT assay was performed as described in the methods section 3.4. The culture supernatant were added to the Maxisorp® ELISA 96 well plates (Nunc) to perform MIP1α ELISA using the human Duoset Human MIP-1α ELISA kit (R&D systems). Culture supernatants were added in doubling dilution after initial calibrations with neat and 1:10 , 1:50 and 1:100 dilutions. The 96 well plates were read at the end of the protocol with fluorescence intensity (FI) per well was read at λex488nm/λem528nm using a FLx800 multidetection microplate reader (Biotek Instruments, USA). FI data were collected using Gen5 1.07 software. The concentrations of MIP-1α were estimated by reading off the standard curve.

IL-6 ELISA: HS5 stromal cells were plated on the 96 well plates overnight at a density of 2 x 10^4 cells per well. Once the stromal cells were fully covering the base of the wells, MM1S and MM1R cells were plated at a density of 2 x 10^5 cells ml in 96 well plates with and without drugs for 24 hours. Dasatinib 150nM, Dexamethasone 1uM and combination of Dasatinib and

dexamethasone were used. The supernatants were taken after 24 hours and stored in -20°C until experiments were performed. The supernatants were added to the Maxisorp® ELISA 96 well plates (Nunc) to perform IL-6 ELISA using the human Duoset Human IL-6 ELISA kit (R&D systems). Culture supernatants were added in doubling dilution after initial calibrations with neat and 1:10, 1:50 and 1:100 dilutions. The 96 well plates were read at the end of the protocol with fluorescence intensity (FI) per well was read at λex488nm/λem528nm using a FLx800 multidetection microplate reader (Biotek Instruments, USA). FI data were collected using Gen5 1.07 software. The concentrations of MIP-1α were estimated by reading off the standard curve.

EFFECT OF DRUGS ON PLASMA CELLS

4.1 Introduction

4.1.1 Cell Lines and models

Plasma cells are end stage differentiated B cells. Myeloma cells obtained from patients bone marrow separated by immunomagnetic separation fail to survive for long periods despite growth factors and fortified human serum. Therefore most of the laboratory work utilises human myeloma cell lines. Myeloma patients develop secondary plasma cell leukaemia during end stages of their disease and most of the cell lines developed have utilised this source to culture human myeloma cell lines *in vitro.* U266 and RPMI 8226 were the first human cell lines developed. To date 81 myeloma cell lines have been developed from 73 patients with terminal features. Myeloma cell lines have a significant genomic heterogeneity as observed in patients[156]. Presence of high risk cytogenetic features such as t(4;14) and del 17p continue to remain prognostic in the era of novel agents. Some of these cell lines harbour these specific high risk genomic aberrations[156]. Therefore drugs and other therapeutic interventions should be tested on a panel of cell lines. They serve as a useful resource to study the effect of drug combinations and biological features of the disease. Human myeloma cell lines also differ in their requirement for growth factors to maintain these cells in long term cultures, such us IL-6 dependence in INA-6 and XG-1 cell lines. Caution has been raised by Drexler et al who have reported on so called “false” myeloma cell lines used in several publications over the years. He defines them as EBV transformed lymphoblastoid cell lines with immunophenotypic and genotypic characteristics of lymphoma cells [157]. This underpins the importance of

choosing a panel of cell lines and growing them in adequate conditions to ensure that they retain parental characteristics. Myeloma cell lines with differential response to steroids which is one of the backbone of myeloma therapies, is an extremely useful tool to study drug combinations. Greenstein et al have developed cell lines from a single MM patient. Three separate cell lines were established that parallel the progression of the disease. These three cell lines, designated MM1.S, MM1.RE, and MM1.RL, can be distinguished on the basis of their sensitivity to steroid hormones such as glucocorticoids[158].

Myeloma models have been developed over the years particularly to look at biological aspect of mutations driving the disease, bone disease, drug effects in immunocompromised mice injected with myeloma cell lines. 5T myeloma cells that arose spontaneously in aged, inbred C57BL/KaLwRij mice and are propagated by the inoculation of these myeloma cells into syngeneic mice have been developed. 5T33 myeloma cell lines have been generated characterised and maintained in syngeneic animals by Radl et al at Leiden[159]. More recently using lentiviral transduction eGFP expressing 5T33 cell line was developed and described by Alici et al. They used the fluorescent marker to map the homing and quantify tumour load in the mice[160].

Primary cells maintained in pooled plasma or in cultures fortified with IL-6 have been used to study effects of the drugs in vitro. Efforts are on to improve the ex vivo cultures of myeloma cells to better understand the effects of drugs on primary myeloma cells from patients with relapsed disease.

4.1.2 Drug Exposure

The underlying principles of drug exposure are based on an understanding of the causal relationships between exposure and effect. When the cell lines are exposed to drug at varying concentration the classical dose response curve which is sigmoid shaped is generated. The lowest dose at which there are no effects observed is called No Observable Effect Level (NOEL). The dose at which 50% of cells apoptose or die would be classified as IC 50, in other words, it is the half maximal (50%) inhibitory concentration (IC) of a substance. It is important the concentrations studied in the lab are clinically achievable. The main stumbling for drug therapy is toxicity and concentrations safely achieved in Phase I trials should guide these experiments

When drug combinations are studied together, serial concentrations of drug are added to the cell lines and a dose response curve is generated. The combined effect of the drugs could either be additive, antagonistic or synergistic. Calcusyn is the software employed in my thesis to study these effects. Calcusyn is a software which analyses effect of drug combinations and is able to automatically quantify phenomena such as synergism and inhibition. The software performs multiple drug dose-effect calculations using the Median Effect methods described by T-C Chou and P. Talalay. A Combination Index (CI) value of <1.0 indicates synergy and >1.0 is additive.

Effects of drugs on cell lines and primary cell should be validated in other systems with other read outs. For example in studying cell proliferation, employing MTT assays, cell counts and trypan blue estimation to look at viability. This will enhance the validity of the results. I have performed cell cycle analysis, expression of Annexin V and 7-AAD to look at proportion of

early apoptotic cells, necrotic cells and also examine the number of cell in Sub G0 phase (late apoptotic cells).

4.1.3 Soluble factors

Myeloma cells secrete soluble factors which help autocrine growth along with paracrine effects on the accessory cells in the microenvironment. The tumour cells activate the stroma by producing and secreting cytokines and growth factors, matrix metalloproteinases (MMPs). Myeloma cells can produce and secrete a variety of cytokines and growth factors into the MM BM microenvironment, including: IL-6, IGF-1, VEGF, TNF-α, SDF-1α, TGF-β, bFGF, MIP-1α, SCF, HGF, IL-1β, IL-3, IL-10, IL-15, and IL-21, as well as Ang-1 and matrix metalloproteinases (e.g. MMP-2 and MMP-9) [54]. IL-6 is the major growth and survival factor in MM cells, and also confers drug resistance to dexamethasone. VEGF and IGF -1 also secreted by plasma cells induce both autocrine and paracrine effects in the microenvironment driving tumour resistance and angiogenesis.

IL-6 and MIP 1α are the two key cytokines measured in the experiments described this chapter. IL-6 is a key cytokine with pleiotropic effects on the plasma cell clone. Serum levels of IL-6 reflect disease severity in multiple myeloma patients. The levels of IL-6 are low in patients with smouldering disease or MGUS[161]. MIP-1α plays an important role in mediating bone disease in myeloma patients[162]. The serum level of MIP 1α is elevated in myeloma patients with active disease[163].

4.2 Aims

The aim of the experiments reported in this section of thesis is to determine whether Dasatinib, dexamethasone and the combination inhibit cell proliferation. If the drugs inhibit myeloma cell proliferation the next set of experiments will be to determine whether their effect of the drug combination is additive, antagonistic or synergistic. The levels of key cytokines IL- 6 and MIP 1α will be measured to observe whether drug exposure inhibits secretion of these cytokines which provide autocrine growth and paracrine effects. Induction of apoptosis will be analysed by studying the expression of Annexin V. Cell cycle analysis will be performed to confirm the induction of apoptosis in these cell lines and to look at the proliferation fraction of the cells exposed to drugs.

4.3 Effect of drugs on proliferation of human myeloma cell lines

Human myeloma cell lines MM1S, MM1R, RPMI 8226, U266, ARH 77 and murine myeloma cell lines 5T33 and 5T33 GFP were chosen for studying the effect of drug exposure. To determine the actual cell numbers required to study the proliferation over a 96 hour period, a growth curve was performed using the MTT assay.

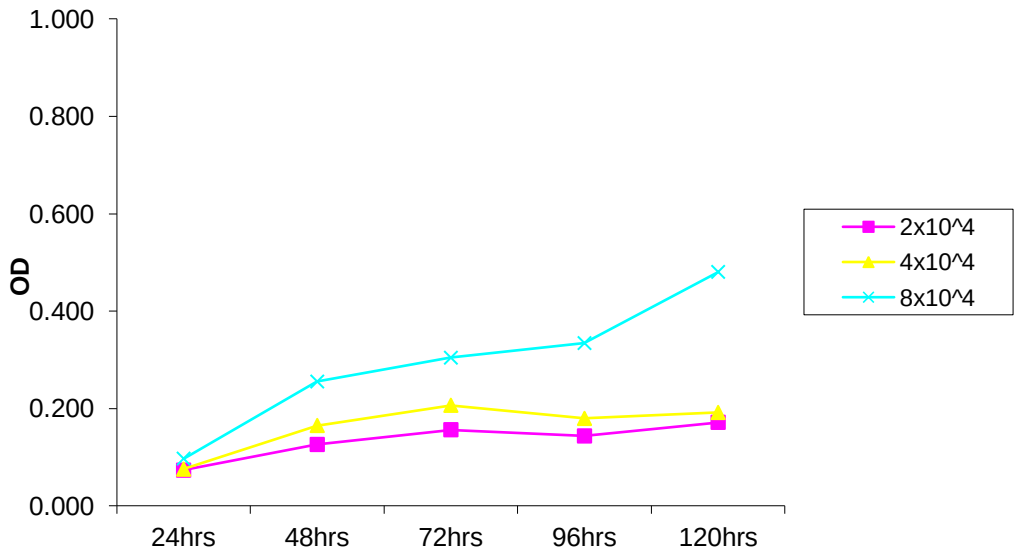
4.3.1 Growth curve

MM1S and MM1R cell lines were seeded in 96 well plates in the following cell numbers in media (Fig 1). Cell numbers 2 $x10^4$, 4 $x10^4$ and 8 x 10^4 per well in 200ul of culture medium were seeded in 5 plates. These plates were analysed by MTT assay as described in the methods and materials section including a control well (blank) for control of the background of OD emission in empty wells. Optical density values presented in Graphs 1-3 are after subtraction from blank values (control). The optical density values were plotted in Y axis and the time period in X axis. MM1S cells plated at a concentration of more than 8 x 10^4 cells per well (4 x 10^5 cells/ml) showed a proliferative response and this was used as initial seeding concentration for further experiments. MM1R cells plated at a density of more than 2 x 10^4 cells/well (2 x 10^5 cells per ml) showed a serial growth over 5 days and was used as initial seeding density of cells when plated alone or in coculture in further experiments described in the thesis. ARH77 cells, RPMI 8226 and U266 were plated at the same densities as described above over a 5 day period (Fig 2). The initial cell density levels of 4 $x10^4$ cells per well of media was adequate to provide exponential growth in all three cell lines. A higher cell density of 8 x 10^4 cells

per well media showed no growth in ARH77 and RPMI8226 cell lines. This is probably due to the media being depleted of nutrients within the first 24 hours due to the higher concentration of cells.

Fig 1. Growth curve of MM1S (A) and MM1R (B) cells over a 5 day period.

A



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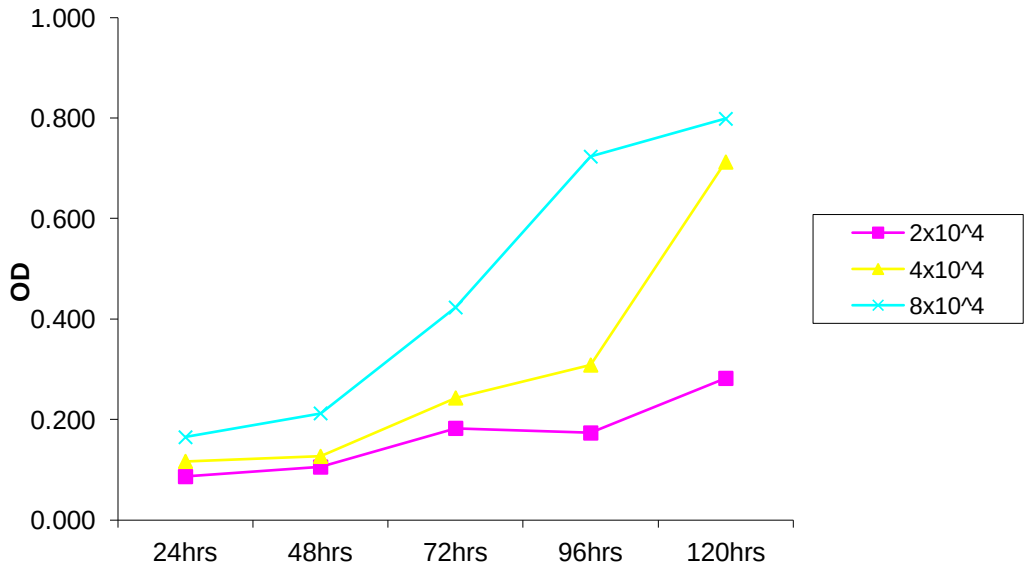
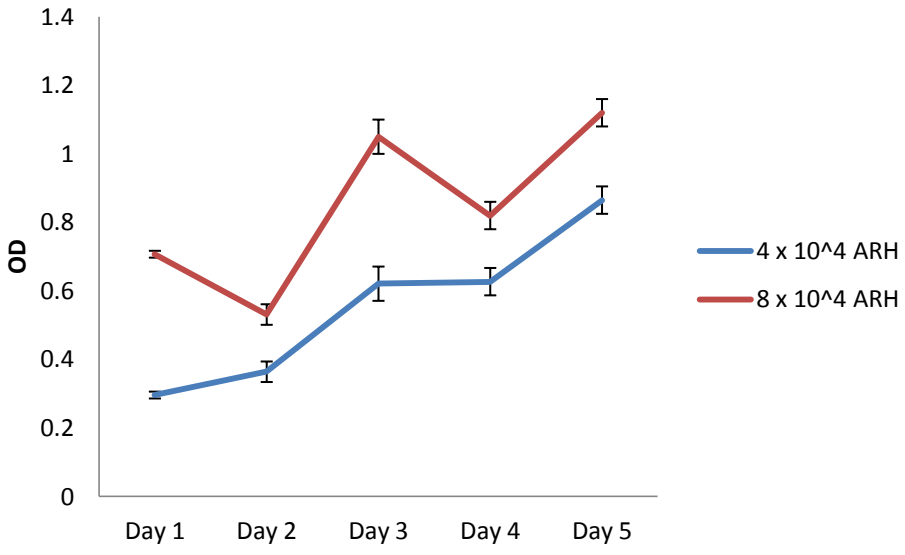
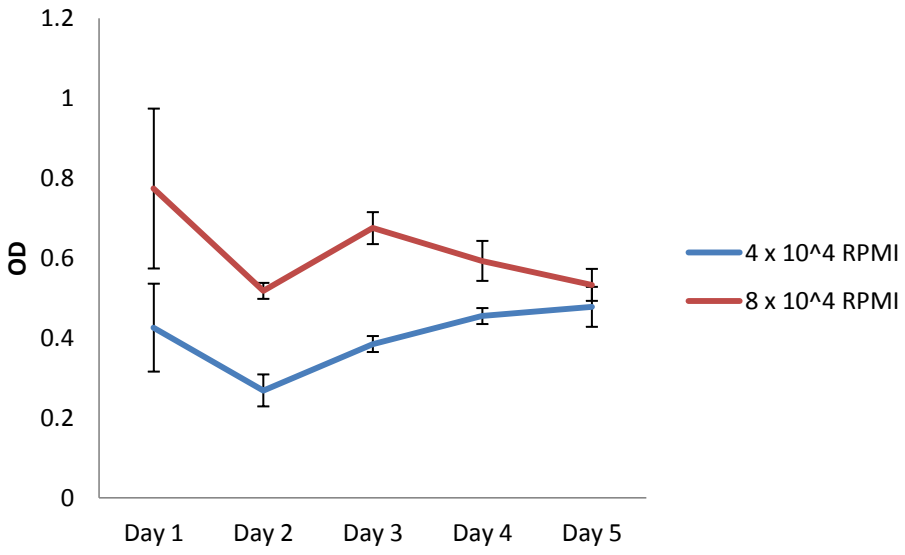
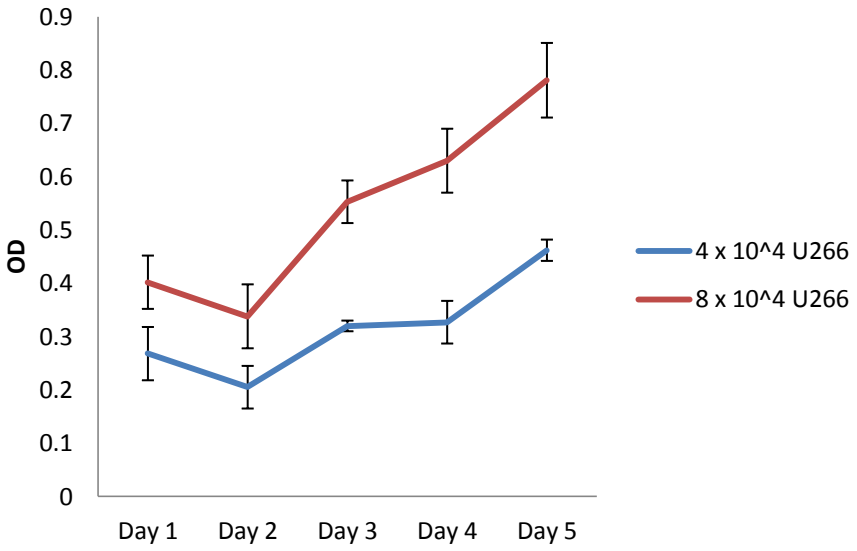


Fig 2: Growth curve of ARH77, RPMI 8226 and U266 human myeloma cell lines over a 5 day period.



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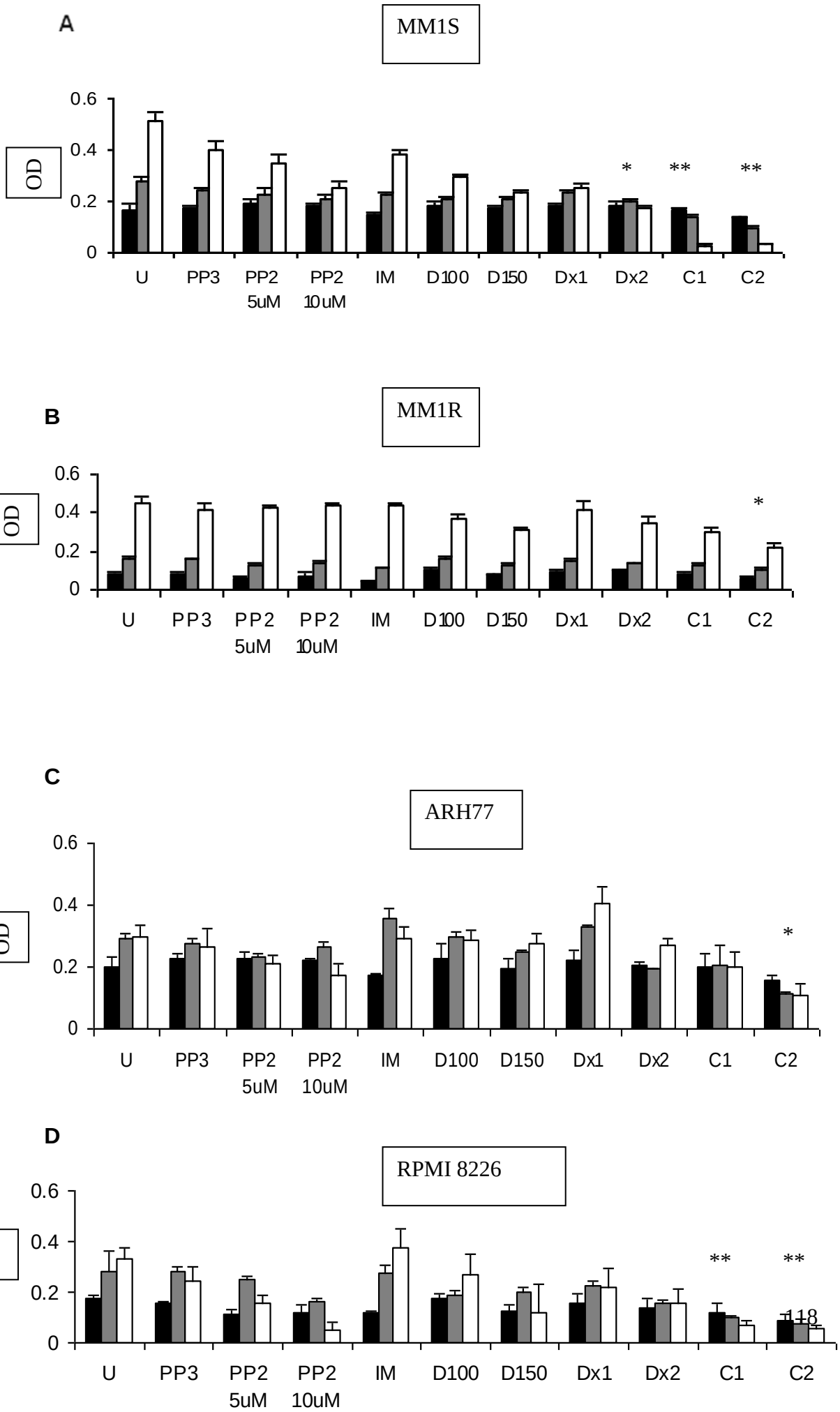
4.3.2 Proliferation of human myeloma cell lines

Human MM1S, MM1R, RPMI8226, ARH 77 and U266 cells were either left untreated or treated with Dasatinib used at 2 different concentrations 100nM and 150 nM. Concentrations above this were not chosen as the maximum serum concentration achieved in phase I studies was 180nM[164]. Subsequent to these initial studies, clinical efficacy with tolerable side effects was observed at a lower dose compared to those used in Phase I studies [143]. Dexamethasone was used at 2 concentrations 1uM and 2 uM as reported in other studies[165]. Combination of dasatinib and dexamethasone was used at 2 dose levels, 100nM + 1uM and 150nM + 2uM to inhibit proliferation of myeloma cell lines. PP3 an inactive Src analogue (Calbiochem, UK) which serves as control for Src inhibitor PP2 (Calbiochem, UK). PP2 is a commercially available Src inhibitor with chemical name (4-amino-5-(4-chloro-phenyl)-7-(*t*-butyl)pyrazolo[3,4-*d*]pyrimidine was initially reported as highly selective for Src family kinase[166]. Subsequently it has been shown that PP2 down regulates both c-kit and Abl kinase activity[167]. Cells were also treated with Imatinib mesylate, a tyrosine kinase inhibitor which down regulates abl kinase activity. Cell lines left untreated continued to proliferate as evidenced by increased cell numbers on the MTT assay. Treatment with dexamethasone strongly inhibited proliferation of MM1S, RPMI8226 and U266 cells even at the lower concentration (Figure 3). MM1R, ARH77 cells were resistant to dexamethasone. Dasatinib on its own even at the higher concentration of 150nM had a more modest inhibition of proliferation in MM1S and RPMI 8226 cells with almost no effect on the other human myeloma cell lines.

Combination of dasatinib and dexamethasone inhibited proliferation of all human myeloma cell lines tested but clearly cells which were dexamethasone sensitive showed a more profound reduction in proliferation compared to dexamethasone resistant cells (Figure 3). This was observed at both levels of combined dose used. PP2, inhibitor of Src kinase inhibited proliferation of MM1S, RPMI 8226, U266 human myeloma cell lines. The differences observed between PP2 and dasatinib could be explained partly by concentration of dasatinib used in these experiments. The differences could also be due to the spectrum of different tyrosine kinases inhibited by both drugs. Imatinib mesylate showed almost no effect on proliferation in human myeloma cell lines.

Figure 3: Growth inhibition of myeloma cell lines by combination of Dasatinib and dexamethasone. Cell numbers in myeloma cell line cultures were estimated using MTT assay at 6 hours ■ day 1 ▒ and day 5 ▢ post-plating. Cells were left untreated (U) or treated with 10uM PP3, 5uM &10 uM PP2, 10uM Imatinib (IM) 100nM and 150 nM Dasatinib (Das100, Das150) , 1uM and 2 uM Dexamethasone (Dx1 & Dx2) and combination of 100nM Dasatinib and 1uM Dexamethasone (C1), combination of 150nM Dasatinib and 2uM Dexamethasone (C2) . Histograms A) MM1S B) MM1R C) ARH 77 D) RPMI 8226 E) U266. Cell lines were Human derived (A-E). Histograms show Mean +SD. * $p < 0.05$ Student t- test versus control untreated ** $p < .001$. Student t-test versus untreated cells.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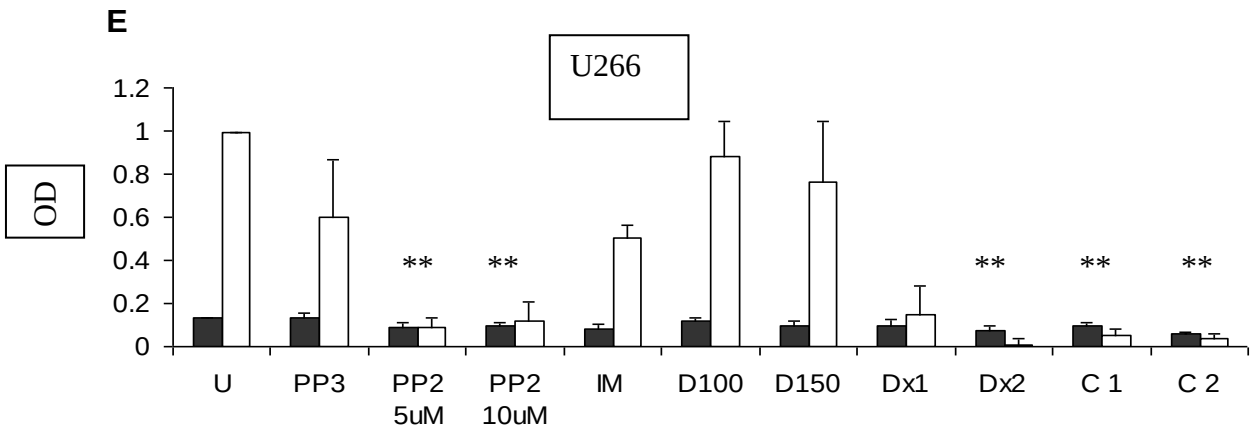
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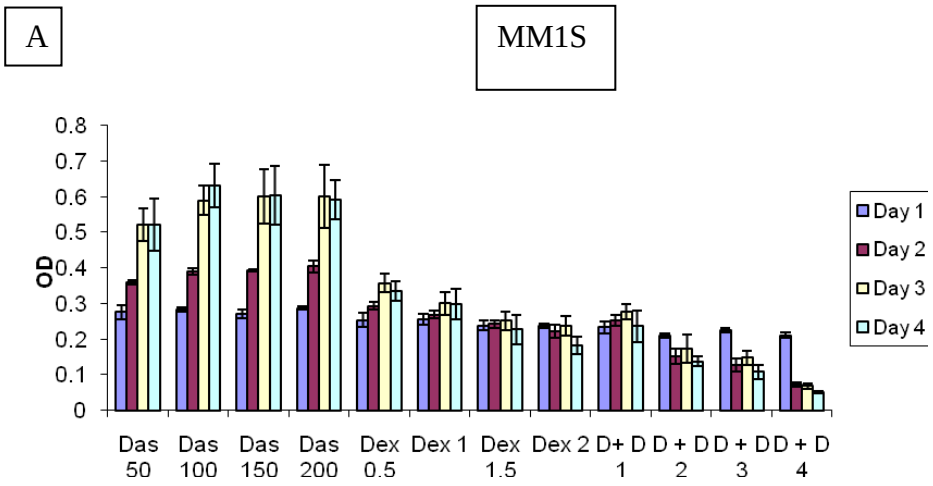
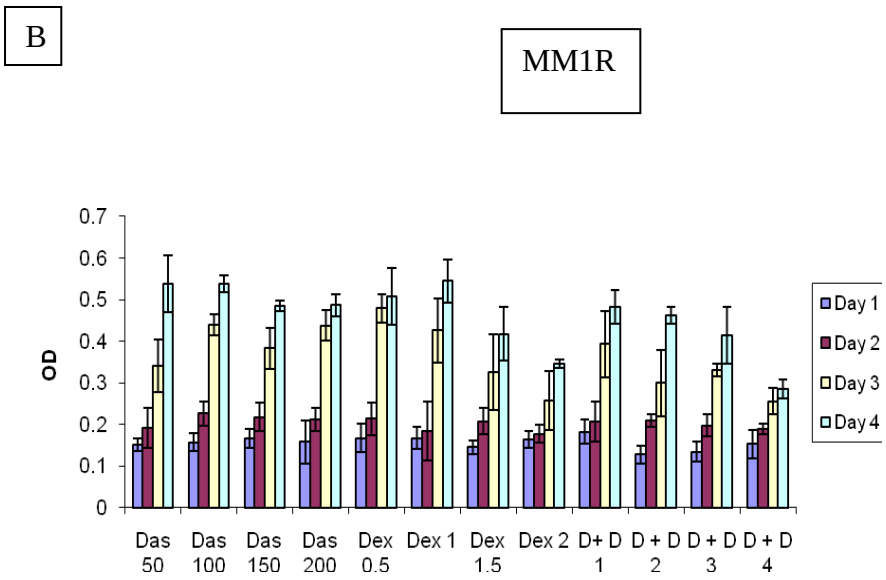
4.3.3 Synergy Assay

Dasatinib and dexamethasone combination inhibited proliferation of human myeloma cell lines tested. To establish whether these effects are additive, antagonistic or synergistic proliferation assay was performed at various drug concentrations of dexamethasone alone, dasatinib alone and the combinations. MM1S (Dex sensitive) and MM1R (Dex resistant) cell lines were exposed to Dasatinib at concentrations of 50nM, 100nM, 150 nM and 200nM with dexamethasone 0.5uM, 1uM, 1.5uM and 2uM respectively. Cell lines were also treated with paired combinations of dasatinib and dexamethasone at all four dose levels. MM plasma cells continued to proliferate in presence of Dasatinib concentrations between 50nM and 200nM. As expected MM1S failed to proliferate when treated with dexamethasone whereas only the highest concentration of dexamethasone inhibited proliferation of MM1R in modest amounts. The combination inhibited proliferation of both cell lines with a clear dose effect (Fig 4).

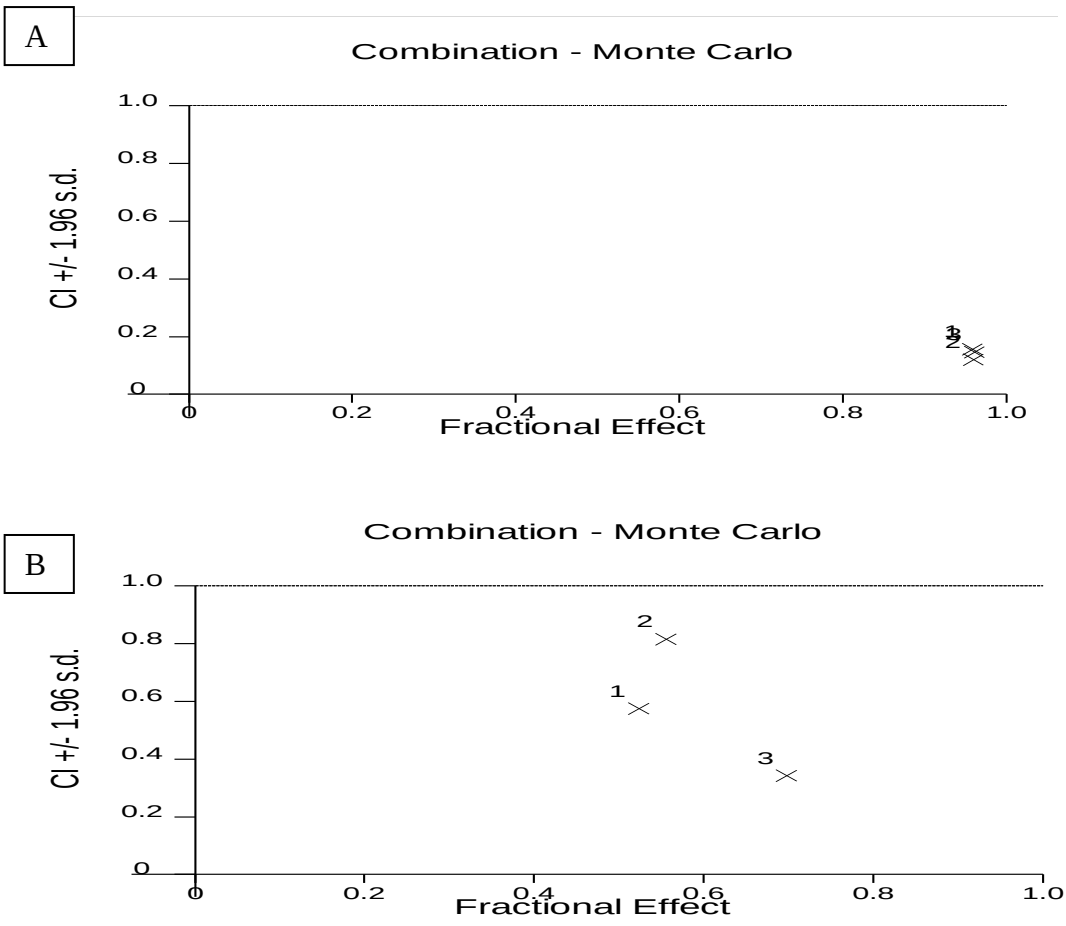
Figure 4: Growth inhibition to varying combinations of Dasatinib, dexamethasone. Cell numbers in myeloma cell line cultures were estimated using MTT assay on day 1 , day 2 , day 3 and day 4 , post-plating. Cells were treated with 50nM, 100nM, 150 nM and 200nM Dasatinib (Das50, Das100, Das150, Das 200) or 0.5uM, 1uM, 1.5uM and 2 uM Dexamethasone (Dex 0.5, Dex1, Dex 1.5 & Dex2) or combination of 50nM Dasatinib and 0.5uM Dexamethasone (D+D1), 100nM Dasatinib and 1uM Dexamethasone (D+D2), 150nM Dasatinib and 1.5uM Dexamethasone (D+D3), combination of 200nM Dasatinib and 2uM Dexamethasone (D+D4) . Histograms A) MM1S B) MM1R. Histograms show Mean +SD





The data obtained from the MTT assay were input into the Calcusyn software. The software is based on the Chou-Talalay method. The resulting combination index (CI) theorem of Chou-Talalay offers quantitative definition for additive effect (CI = 1), synergism (CI < 1), and antagonism (CI > 1) in drug combinations [168]. The above OD data obtained with combinations of dasatinib (up to 150nM) and dexamethasone (up to 1.5uM) were analysed by Calcusyn 2.0 software (Fig 5). In both MM1S and MM1R myeloma cell lines, all the examined dose level combination s of Dasatinib and dexamethasone showed synergy in inhibiting cell proliferation.

Figure 5: Synergy assay: Calcusyn software output for MM1S (A) and MM1R (B) cell lines showing 3 different combination dose levels 1- 50nM Dasatinib and 0.5uM Dexamethasone (D+D1), dose level 2 - 100nM Dasatinib and 1uM Dexamethasone (D+D2), dose level 3- 150nM Dasatinib and 1.5uM Dexamethasone (D+D3). The values are less than 1.0 confirming synergistic effects in both cell lines.



4.4 Apoptosis measurement and cell cycle analysis

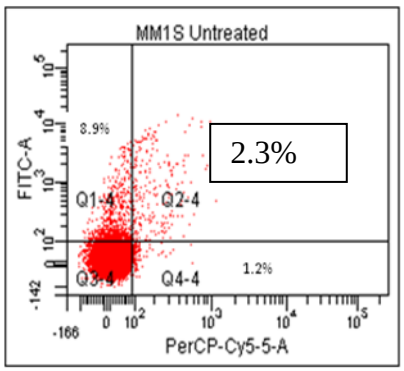
Exposure to the combination of dasatinib and dexamethasone inhibited the proliferation of the myeloma cell lines. To understand whether inhibition of cell proliferation was cytostatic or correlated with increased cell death, cell lines were plated at the same density in parallel to proliferation assays in 24 well plates with and without drugs. On the days of proliferation analysis, cells were stained for 7-AAD and Annexin V in parallel for flow cytometric analysis to determine the percentage of apoptotic and necrotic cells. Phosphatidylserine is translocated to surface of plasma membranes on myeloma cells and Annexin V, an early marker of apoptotic cells preferentially binds to negatively charged Phosphatidylserine in the presence of Calcium. 7-AAD expression is a marker of necrotic cell death and late apoptotic cells. 7-AAD is an analogue of actinomycin D and binds to DNA of late apoptotic cells (7- AAD positive Annexin V positive) and necrotic cells (7 –AAD positive and Annexin V negative). In MM1S cells, there was 5- 10% apoptotic fraction in untreated cells at baseline. Upon treatment with Dasatinib 150nM after 4 days there was no significant increase in apoptosis or necrosis (Fig 6A). Treatment with 1uM dexamethasone induced high levels of apoptosis. The combination of dasatinib and dexamethasone induced significant apoptosis and cell death with up to 70% of cells staining for both 7-AAD and Annexin V and 25 % staining for Annexin V alone. In MM1R cells there was a small increase in AnnV/7-AAD positive fraction from 2.4% to 5.7% (Fig 6B).

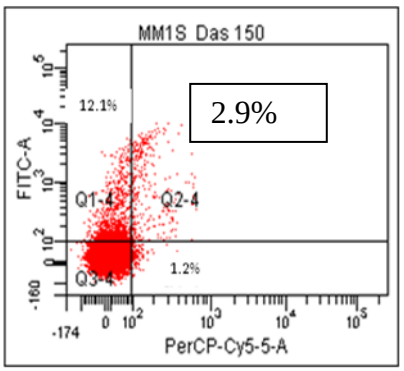
Fig 6: Flow cytometric analysis of apoptotic and necrotic fraction in the presence of drugs. MM1S (A) and MM1R (B) cells left untreated (U), or treated with dexamethasone 1uM(Dex), Dasatinib 150nM (Das) and combination of dasatinib and dexamethasone (D+D). Flow cytometric analysis with Annexin V staining (FITC) in Y axis and 7-AAD staining (PerCP-Cy5.5A) in X axis.

MMIS

A

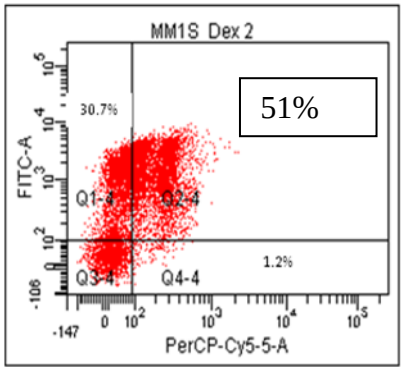
U

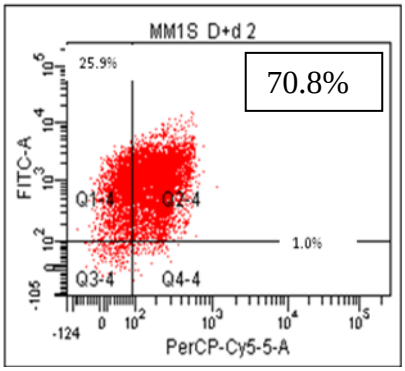




Das

Dex



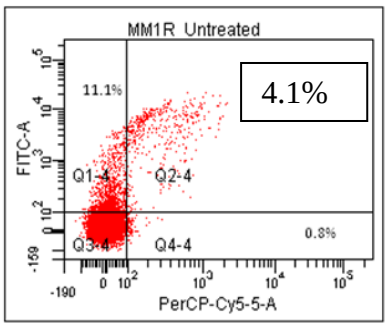


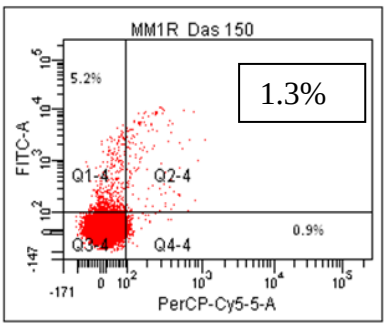
Das +
Dex

MMIR

B

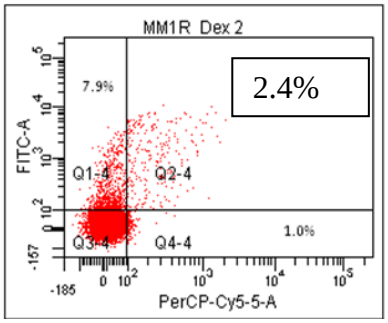
U

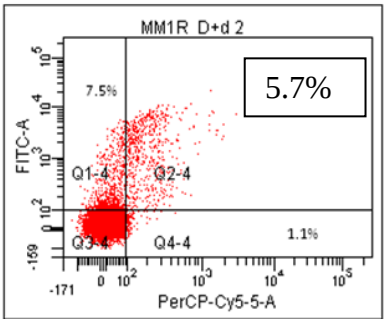




Das

Dex





D+D

Cell cycle analysis:

Determining the relative cellular DNA content can provide a great deal of information about the cell cycle, and consequently the effect on the cell cycle of added stimuli, e.g. Drug treatment. Four distinct phases can be recognised in proliferating cells: the G0/G1-, S- (DNA synthesis phase), G2- and M-phase (mitosis). However, G2- and M-phase, which both have an identical DNA content, cannot be differentiated based on their DNA content. The major limitation to this analysis is the presence of doublets and clumps in the cell suspension. A doublet is formed when two cells with a G1-phase DNA content are recorded by the flow cytometer as one event with a cellular DNA content similar to a G2/M-phase cell. This could lead to a false overestimation of G2/M population. Reduced DNA content in the Sub G0/G1 cell population is a useful way of detecting very late stage apoptotic phenotype cells [169,170]. The limitation of this use is a sizeable proportion of this SubG0/G1 fraction could include cell debris. Human myeloma cell lines were fixed with ethanol and treated with RNase before staining with propidium iodide. Cells were plated in parallel to proliferation assays in 24 well plates with and without drugs to perform cell cycle analysis. MM1S cells upon treatment with the drug combination stopped proliferating as evidenced by absence of G2/M fraction of cells with an increase in Sub G0/G1 fraction of cells (Fig 7, 8). In the MM1R cells although there was a constant fraction of proliferating cells in G2/M phase (25%), an increase in Sub G0/G1 fraction on exposure to drug combination confirming cell death, was observed (Fig 7,8). The proportion of cells in Sub G0/G1 fraction in both ARH77 and RPMI8226 cell lines increase with the combination drug treatment in comparison to dasatinib or

dexamethasone alone. This is was associated with a reduction of proliferating fraction, G2/M phase cells in both cell lines (Fig 9).

Figure 7: Cell cycle analysis. Cell cycle analysis was performed by flow cytometry after fixation with ice-cold 70% ethanol and staining with propidium iodide. The FACS plots shown were performed after 5 days of treatment with dexamethasone or combination of Dasatinib 150nM and dexamethasone 2 uM (Dex 2+ Das 150). MM1S cells are shown on the left panel and MM1R on the right panel. Experiment was repeated three times.

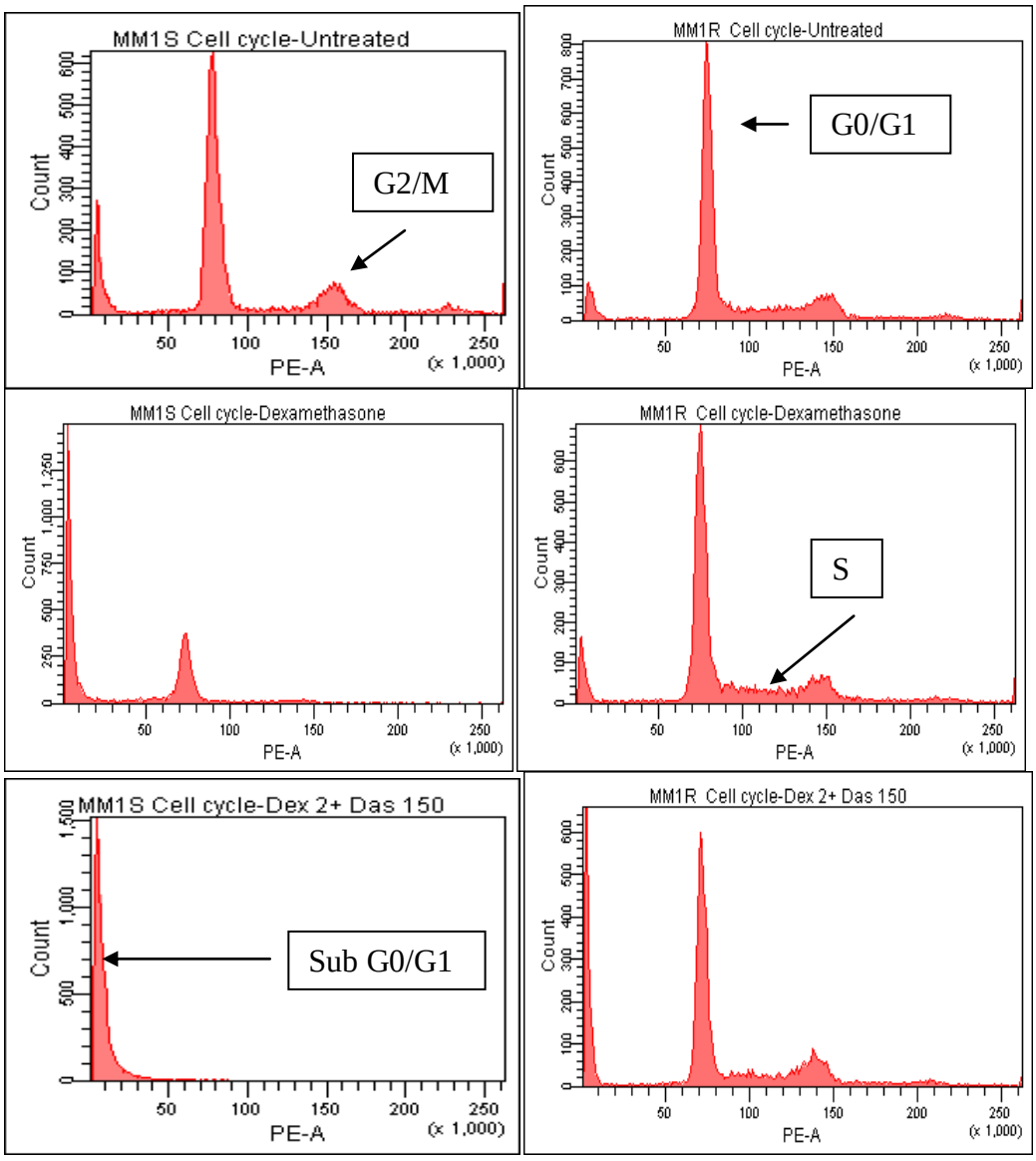
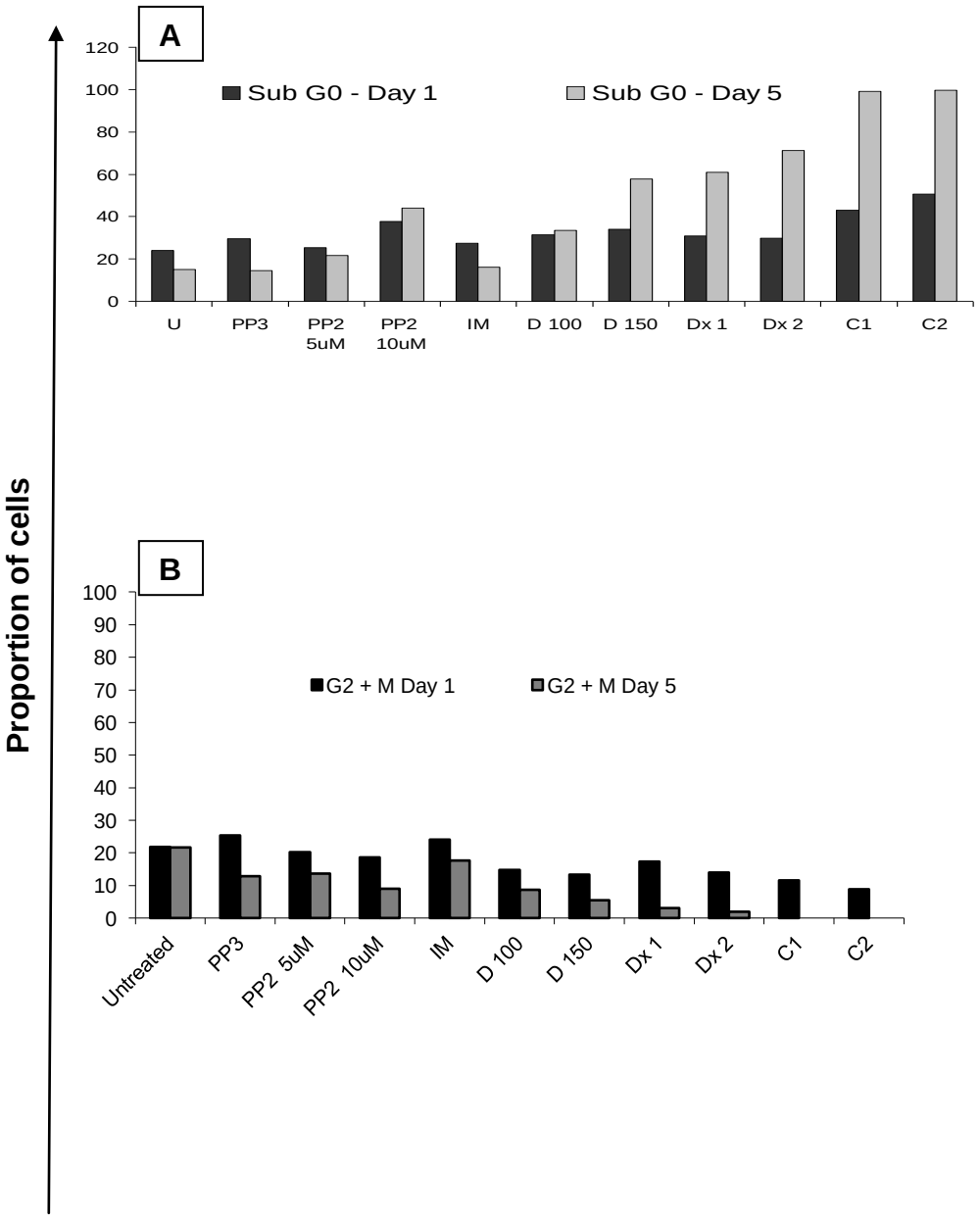


Figure 8: Increased Sub G0 and reduced G2/M populations on treatment with Dasatinib and Dexamethasone. Cell cycle analysis in myeloma cell cultures were performed by flow cytometry after fixation with ice-cold 70% ethanol and staining with propidium iodide on Day 1 ■ and Day 5 □ post plating. Histograms show proportions of nonviable (Sub G0) and proliferative (G2/M) populations of MM1S (A, B) and MM1R (C, D) cell lines in a representative experiment. Experiments were repeated 3 times.



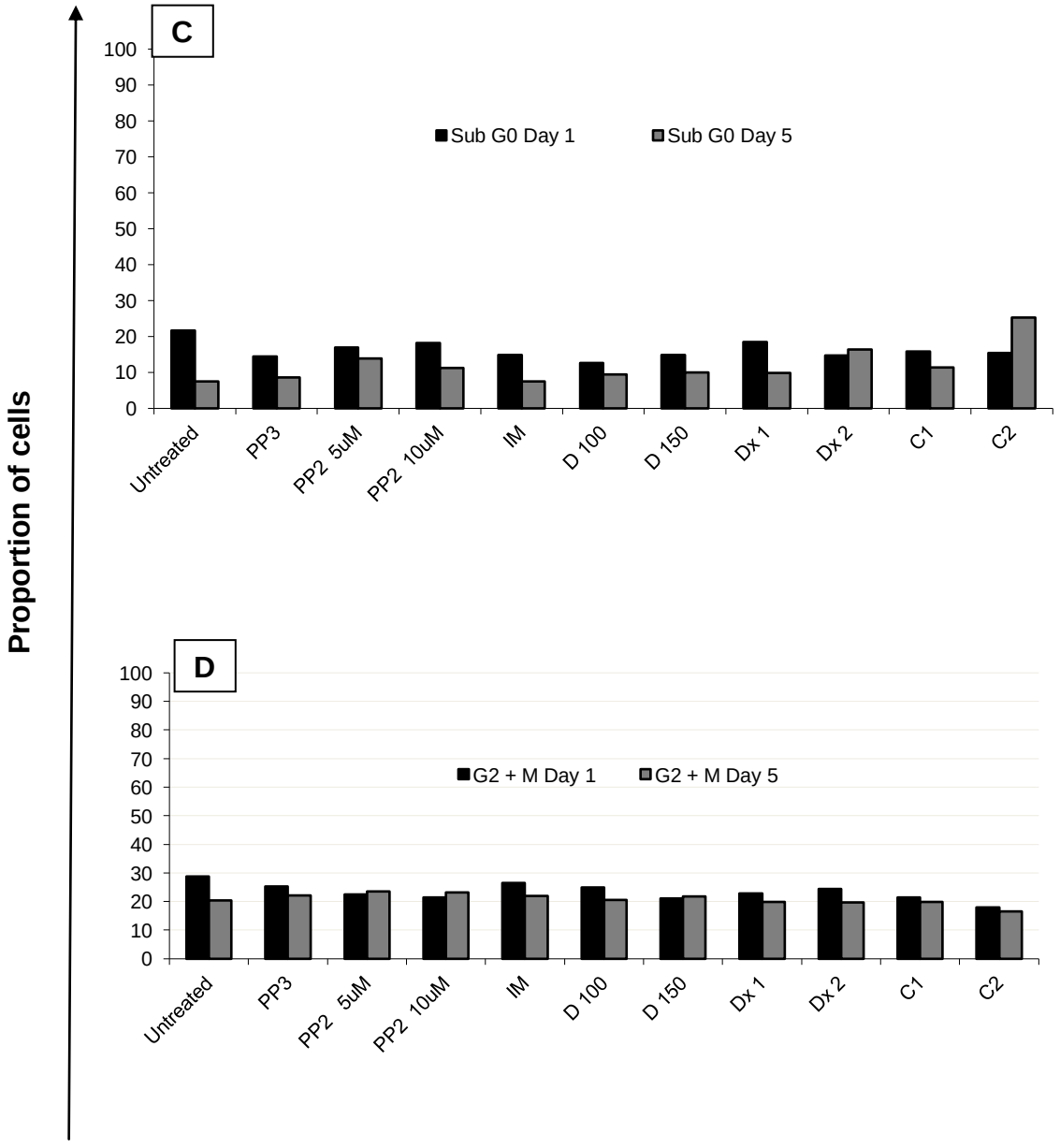
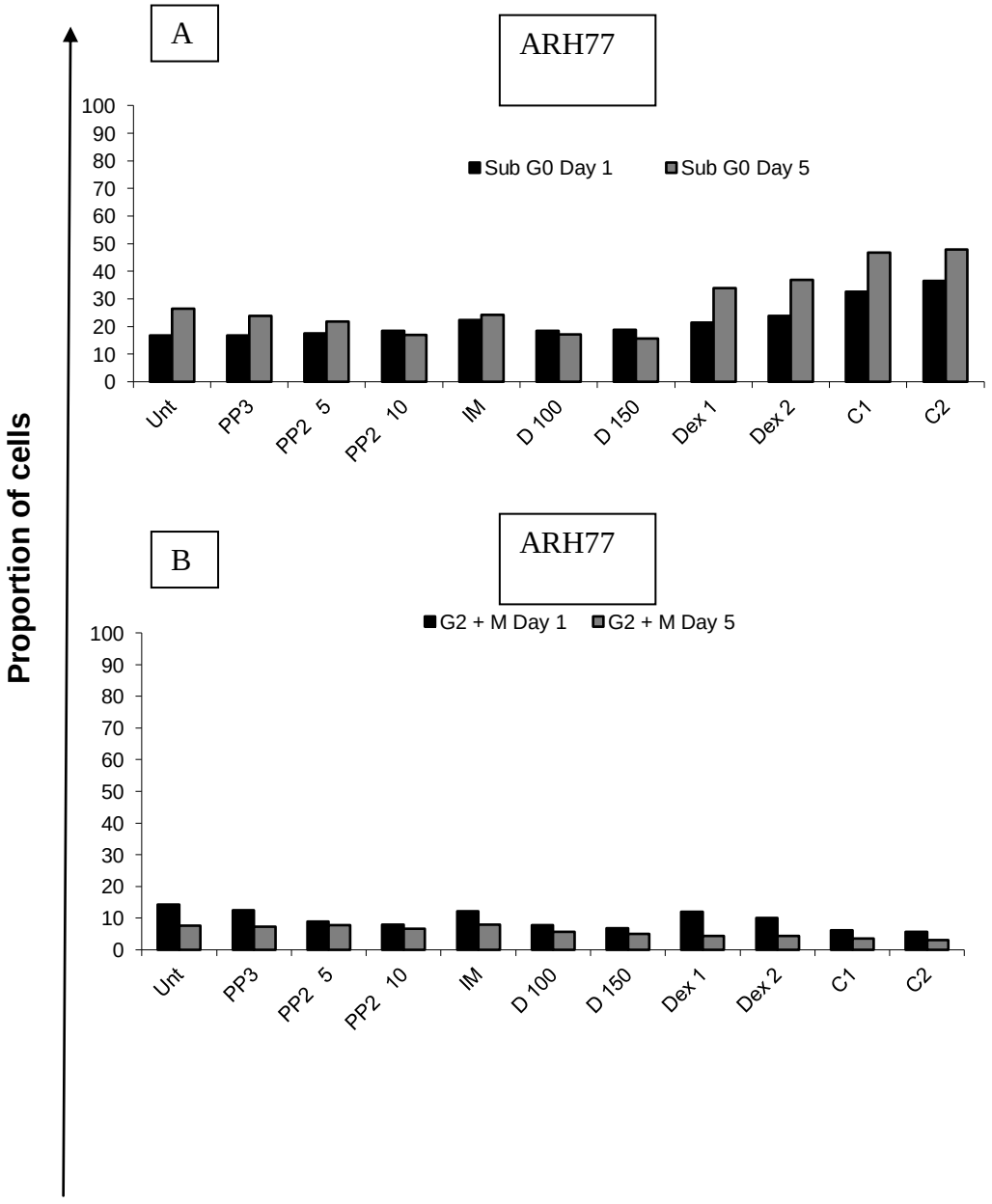
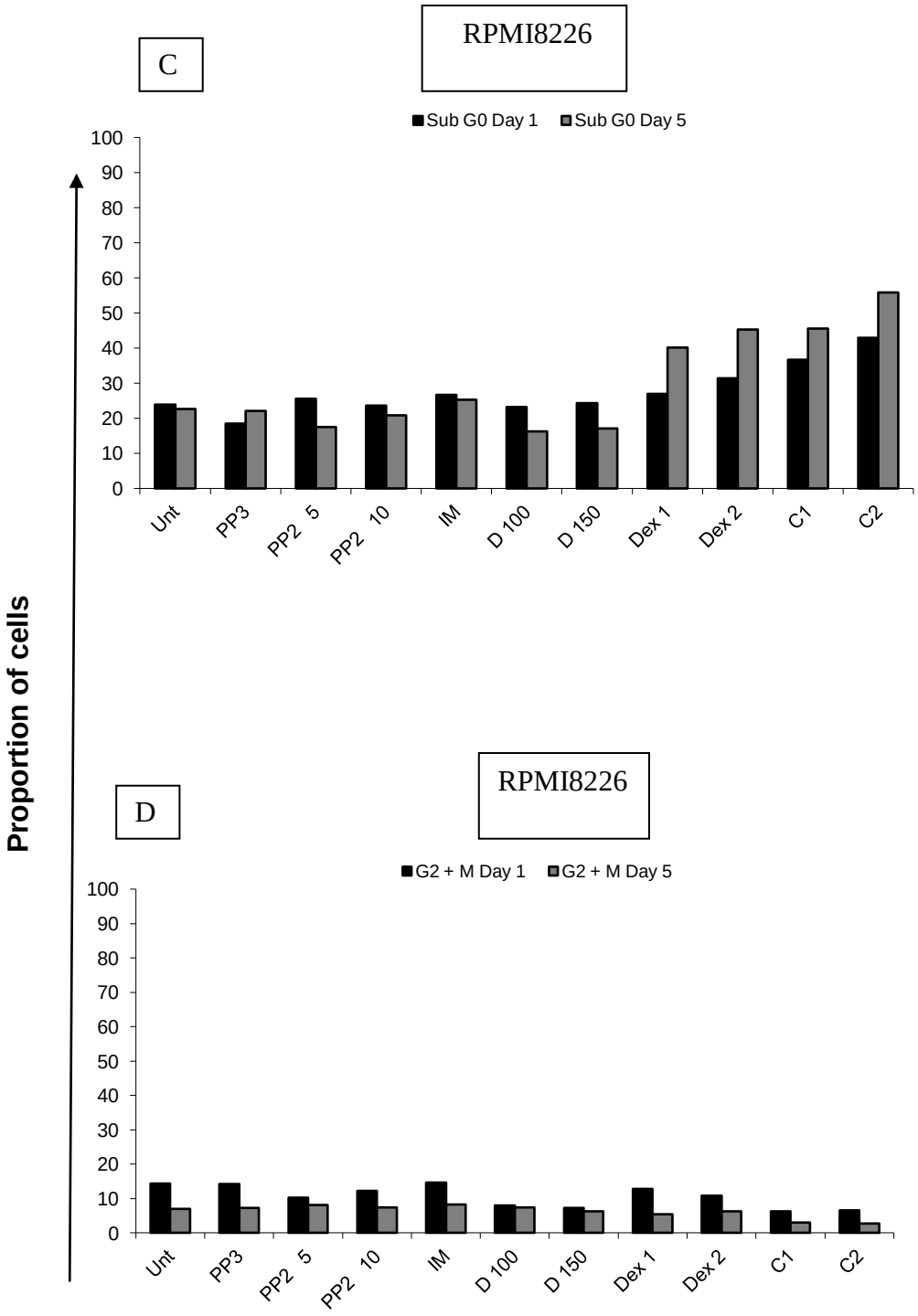

Proportion of cells
C
100
90
80
70
60
50
40
30
20
10
0
Sub G0 Day 1
Sub G0 Day 5
Untreated
PP3
PP2 5uM
PP2 10uM
IM
D 100
D 150
Dx 1
Dx 2
C1
C2
D
G2 + M Day 1
G2 + M Day 5

Figure 9: Cell cycle analysis was performed on ARH77 and RPMI 8226 cells with and without drug treatment. Cell cycle analysis in myeloma cell cultures were performed by flow cytometry after fixation with ice-cold 70% ethanol and staining with propidium iodide on Day 1 (■ and Day 5 (□) post plating. Histograms show proportions of nonviable (Sub G0) and proliferative (G2/M) populations of ARH77 (A, B) and RPMI8226 (C, D) cell lines in a representative experiment. Experiments were repeated 3 times.




C
RPMI8226
Sub G0 Day 1
Sub G0 Day 5
100
90
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10
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PP3
PP2_5
PP2_10
IM
D 100
D 150
Dex 1
Dex 2
C1
C2
Proportion of cells
D
RPMI8226
G2 + M Day 1
G2 + M Day 5
100
90
80
70
60
50
40
30
20
10
0
Unt
PP3
PP2_5
PP2_10
IM
D 100
D 150
Dex 1
Dex 2
C1
C2

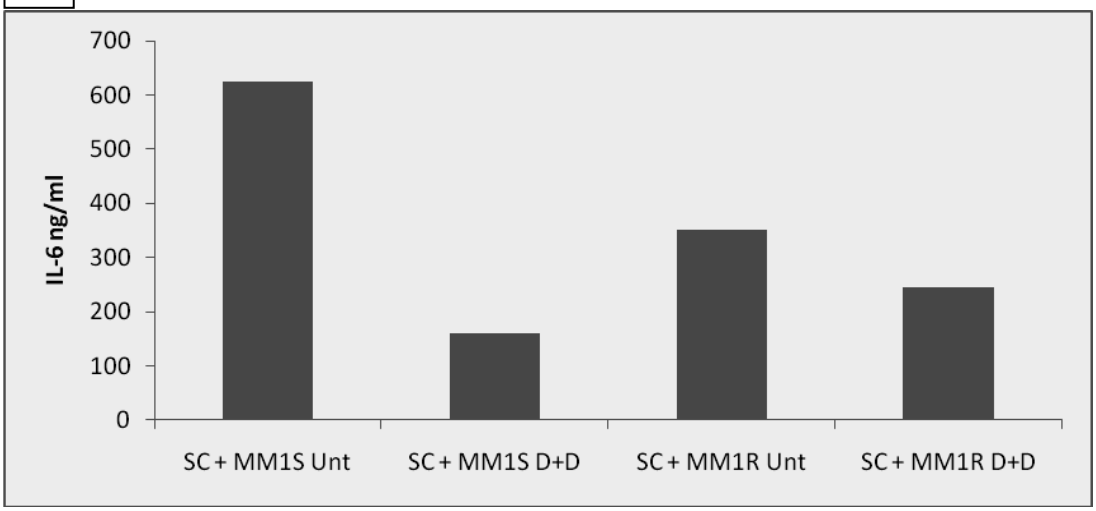
4.5 Effect of drugs on soluble myeloma growth factors

In the in vivo setting when the myeloma cells are exposed to drugs there are key proliferation signals within the microenvironment particularly cytokines enhancing the proliferation and resistance as has been shown with Interleukin 6 (IL-6). Dasatinib and dexamethasone combine to inhibit proliferation of both human and murine myeloma cell lines by inducing apoptosis *in vitro* as shown in the previous experiments. The aim of this set of experiments was to see if the level of the cytokines known to promote survival of myeloma cell, present in the suspension, changed with exposure to drugs. IL-6 is primarily secreted by stromal cells whereas Macrophage inhibitory protein -1 α (MIP -1α) is primarily secreted by the plasma cells. The first set of experiments was done to optimise the number of cells, duration of cultures with media added to blank wells as controls. MM1S and MM1R cells were initially plated at 1 x 10^6 cells/ml with and without drug treatment for 48 hours. Under these conditions, the levels of IL-6 were too low for quantitation. As previously reported, myeloma cells at 5 x 10^5 cells/ml were plated on stromal cells overnight at density 1 x 10^5 cells/ml for 72 hours[171]. IL-6 levels were significantly reduced with dasatinib and dexamethasone combination with a more modest reduction in the cocultures with MM1R cells (Fig 10 A). The levels of MIP 1α dropped significantly with 48 h drug treatment in MM1S cells cultured alone but not in MM1.R cells (Fig 10 B). These decrease in levels of secreted IL-6 and MIP 1α in the supernatants correlated with reduction in cell numbers as previously shown in Figure 4 of this chapter (Fig 10 A). From these results it is not possible to determine if the reduced levels of extracellular IL-6 is due to an effect of the drug on the cells or just due to the decrease in cell numbers. To

study if drug exposure would inhibit release of cytokines from plasma and/or stromal cells, the culture was optimised to short term cultures for 24 hours at a cell density of 2 x 10^5 cells per ml. For IL-6 estimation these cells were plated on stromal cells plated overnight at density of 1 x 10^5 cells per well in 1 ml culture medium. The myeloma cells were left to adhere for 24 hours on stromal cells in media either untreated or treated with dasatinib, dexamethasone or the combination. After 24 hours the supernatants were taken and plasma cell numbers were counted and also estimated using MTT assay. There were no significant differences in cell numbers with drug treatments after 24 hours in both MM1S and MM1R cell lines (Figure 11 A). IL-6 levels decreased with dexamethasone treatment alone and combination of dasatinib and dexamethasone. This was observed in both MM1S and MM1R cell lines (Figure 11 B). This confirms an inhibition in secretion of IL-6 in cocultures of plasma cells and stromal cells by dexamethasone and the combination treatment. MIP-1α secretion by both MM1S and MM1R cells was unaffected by dexamethasone, dasatinib and the combination of both within 24 hours, compared to cells left untreated (Fig 12).

Figure 10: Cytokine levels. IL-6 estimation in ng/ml from supernatants of MM1S and MM1R cells plated at density of 5 x 10^5 per ml on 1 x 10^5 per ml of stromal cells (SC) for 48 hours (A). MIP 1 alpha estimation in ug/ml from supernatants of MM1S and MM1R cells plate at a 1x10^6 cells per ml for 72 hours. Dasatinib and Dexamethasone combination (D+D) reduced IL-6 and MIP 1 alpha levels in both cell lines compared to untreated cells (Unt).

A



B

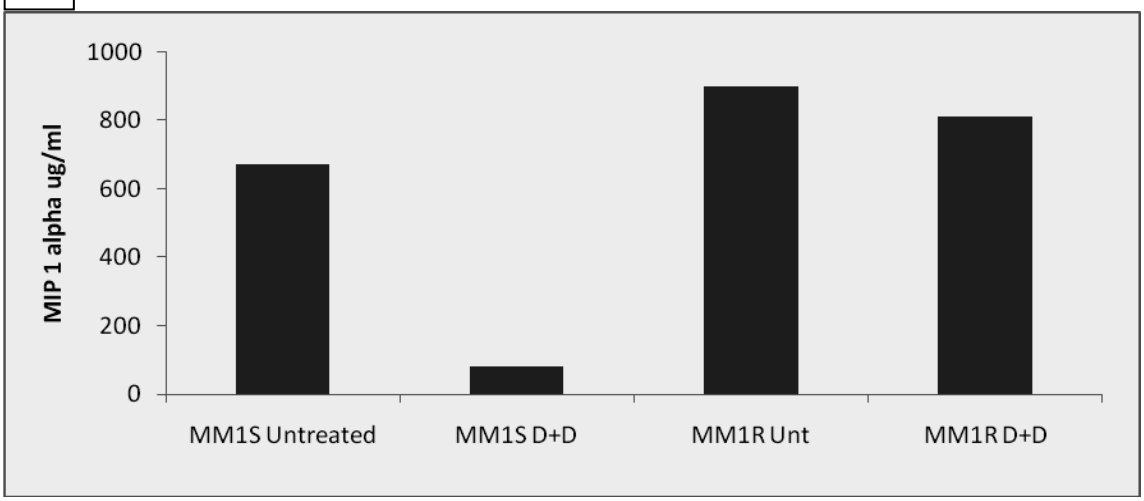
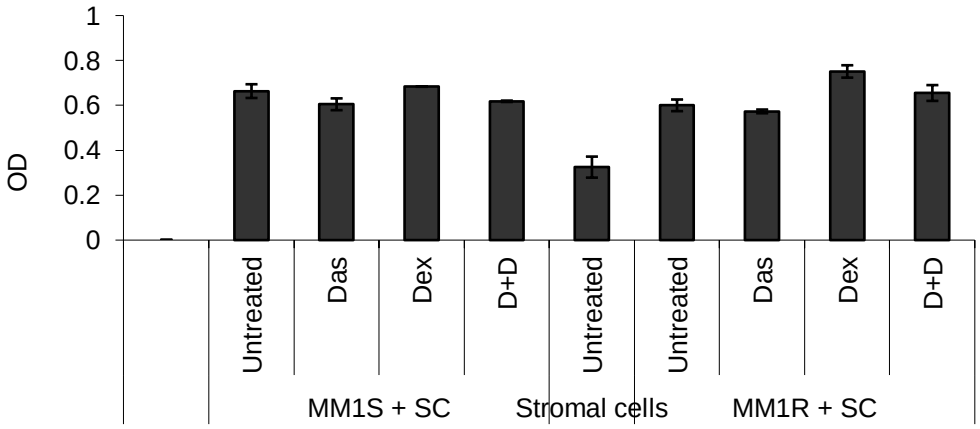


Fig 11: IL-6 levels and cell numbers. Human stromal cells (HS-5) were plated at a density of $1X10^5$ per ml overnight. MM1S and MM1R cells plated at 2×10^5 per ml for 24 hours on stromal cells and left to adhere in media either untreated or treated with Dasatinib (Das), Dexamethasone (Dex) or the combination (D+D). Histograms show cell numbers (A) estimated by MTT assay and IL-6 levels in ug/ml (B) from supernatants after 24 hours. * p <0.05 Student t - test

A



B

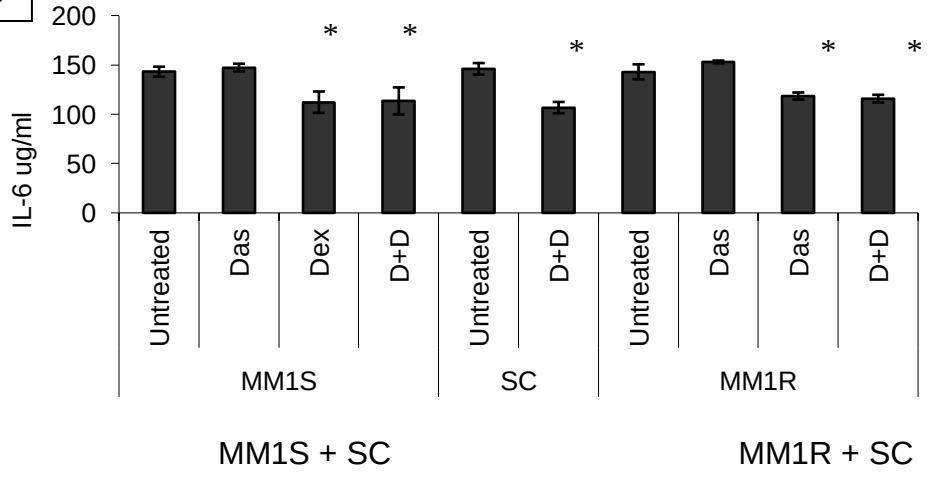
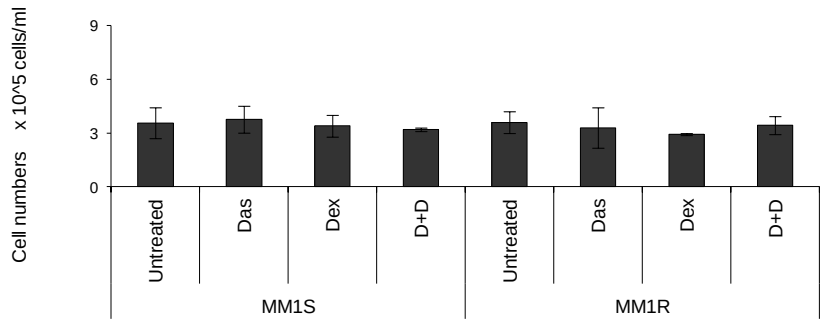
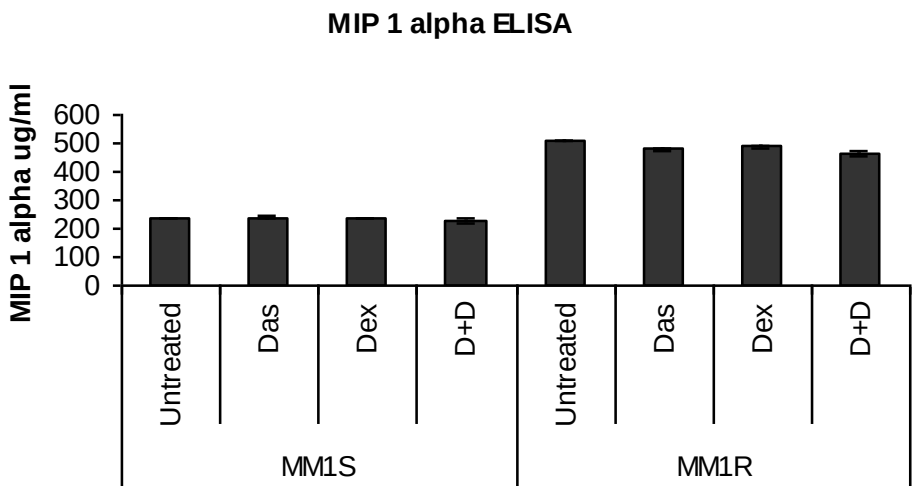


Fig 12: MIP-1α levels and cell numbers. MM1S and MM1R cells plated at 2 x 10^5 per ml for 24 hours in media left either untreated or treated with Dasatinib (Das), Dexamethasone (Dex) or the combination (D+D). Histograms show MIP-1α levels in ug/ml (B) from supernatants and cell numbers (A) were counted.

A



B



The reduction in IL-6 levels aids inhibition of proliferation of myeloma cell *in vivo* by drug treatments. This reduces the paracrine effects on stromal cells, osteoclasts within the microenvironment which is permissive and helps survival of plasma cells. The lack of reduction of MIP-1α in 24 hours shows that secretion remains uninhibited in cell suspensions but with reduction in tumour load induced by cell kill, the total levels of MIP-1α will fall resulting in improvement of myeloma related bony disease.

4.6 Discussion

Dexamethasone based therapies particularly in combination with alkylating agents was mainstay of treatment for myeloma. Although patients respond early they become resistant particularly due to presence of cells in the microenvironment which promote survival of plasma cells, enhance resistance and presence of soluble factors which enhance proliferation. In this section of the thesis, Dasatinib a multitargeted tyrosine kinase, particularly Src kinase was combined with dexamethasone and tested on human myeloma cell lines. The drug combination inhibited proliferation of all the cell lines to variable degrees. The effects were confirmed to be synergistic in both dexamethasone sensitive and resistant cell lines. These findings will be tested in the presence of stromal cells and osteoclast, key cellular partners in the microenvironment in further sections of this thesis. The concentration of dasatinib tested is clinically achievable and on its own has very little effect on the plasma cells. Vij et al showed that single Dasatinib has very little effect in relapsed / refractory myeloma patients [151]. This is predictable based on the results of the experiments described in this section where as a single agent it had very little effect in either inhibiting proliferation of myeloma cells or the induction of cell death. Also the synergism exhibited by the drugs can be exploited clinically, as high doses of dexamethasone used as standard has high degree of non haematological toxicity.

The levels of IL-6 but not MIP -1 alpha secretion fell with treatment using dexamethasone and the combination of dasatinib and dexamethasone. Although Dasatinib alone does not inhibit secretion of IL-6, it down regulates c-Src which is a key intracellular Tyr-kinase and adaptor protein in IL-6

signalling pathway [172]. Dexamethasone is used primarily as an anti-inflammatory agent in clinical practice. Glucocorticoids effectively downregulate tumour necrosis alpha induced cytokine production including IL-6. In the context of myeloma dexamethasone has been shown to reduce IL-6 production by stromal cells [173]. Reduction in IL-6 levels observed was initially thought to be due to enhanced repression of NFκB, but was shown to be due to interference of the activated glucocorticoid receptor with the transactivation potential of the NF-kappaB p65 subunit[174]. Glucocorticoids also up regulate glucocorticoid induced leucine zipper gene in myeloma cells which inhibits NFκB and activated protein-1 (AP-1) stimulated IL-6 production More recently it has been shown that dexamethasone affects the redox state of myeloma cells and transcription factor AP-1 binding activity and resultant IL-6 expression, is dependent on the oxidative state of the cells[175].

There are many possible mechanisms for the observed enhanced pro apoptotic effect of the combination of dasatinib and dexamethasone on plasma cells. Dexamethasone has been shown to induce cleavage of caspase 9 and its activation, whereas dasatinib induced cleavage of caspase 8 and therefore combining well to induce caspase mediated apoptotic cell death [152]. MM1R cells are resistant to dexamethasone because of a truncated glucocorticoid receptor (GR). Sharma et al showed that when cells were Lentiviral transduced with GR, they reversed to become dexamethasone sensitive. In these cells when NFkB is repressed, apoptosis is induced even in cells with truncated GR[165]. They also demonstrated that in non transduced cells upon dexamethasone exposure cells survived despite induction of Bim

and RAFTK phosphorylation. This shows that the dexamethasone resistant cells can be treated with combination of dexamethasone and other agents to induced a response in resistant cell lines. Vacca et al showed PDGFR β, c-Src and VEGF down regulation by dasatinib plays a key role in inhibiting angiogenesis and tumour growth *in vivo*[105]. The inhibition of multiple tyrosine kinases in the plasma cells makes the cell more susceptible to dexamethasone treatment. This effect theoretically could be enhanced *in vivo* because of the effects on the marrow microenvironment with Dasatinib. This observation has major implications *in vivo* where plasma cells adhere to the cellular partners, extracellular matrix proteins and has a favourable soluble milieu along with enhanced angiogenesis in presence of activated osteoclasts. The drug combination could potentially produce significant responses in relapsed patients particularly with active bone disease.

eGFP TRANSDUCTION OF MYELOMA CELL LINES AND DEVELOPMENT OF NOVEL in *vitro* COCULTURE SYSTEM

5.1 Introduction

The development of permissive conditions in the BM microenvironment leads to MM-cell growth, survival, drug resistance and enhanced invasive capacity [54,100,176-178]. Mounting evidence on the role of tumour microenvironment in supporting the growth and survival of myeloma cells makes testing of potential drug treatments *in vitro* in this setting almost obligatory. Recently developed strategies recreate the myeloma tumour microenvironment *in vitro* allowing for detection of myeloma cell proliferation or distribution in bone marrow compartments using cell imaging. However, there is still a need to develop self–contained co-culture methods to apply various laboratory technologies and differentiate the behaviour of myeloma plasma cells from the accessory cells in the tumour microenvironment. Currently, most of the studies evaluate the anti-myeloma potential of drugs on MM cells or different BM microenvironment cells cultured separately. However, in the BM these cells interact with each other and hence, *in vitro* evaluation of the possible anti-myeloma effect of drugs would be performed more accurately using co-culture systems. Although some studies try to address this issue[179-181], the key problem with currently available cell viability assays is its lack of applicability in co-cultures systems, as the signal is generated from all cell populations in culture. Although the luciferase reporter systems partly overcome this problem, by marking the cells of interest, this technique is still restrictive as it allows only evaluation of proliferation and viability of MM cells using luminescence readers [181].

Human [181] and mouse [158] eGFP-MM cell lines have been generated before and used in *in vitro* and *in vivo* studies. The eGFP signal in MM cell lines has

been used to physically separate MM cells in co-culture with stromal cells using FACS sorting for further gene expression profiling [181] as well as for *in vivo* BM homing studies of MM cells [95]. In this chapter, we characterise eGFP-MM cell lines that we have generated. Our data show that our system enables to determine cell numbers using fluorimetry or image analysis with the same sensitivity as standard MTT assay. eGFP expression also enables detection using flow cytometry of apoptotic plasma cell fraction (eGFP positive fraction of co-cultures) in co-cultures with microenvironment cells (eGFP negative fraction of co-cultures), cell cycle analysis and FACS-sorting to apply other techniques such as gene expression profiling [181]. Additionally, the expression of eGFP would allow us to track and/or visualise the morphology of MM cells in co-culture with other BM cells using fluorescent microscopy or live video microscopy. Before embarking on a clinical trial with new agents, validation of the effect of these compounds *in vitro* using eGFP-MM cells lines in the presence of tumour microenvironment provides a self-contained experimental platform for identifying optimal drugs and drug combinations and targeting therapy to individualise treatment.

5.2 Aims

In this chapter, I discuss the generation and validation of eGFP-MM cell lines using colorimetry and fluorimetry. I have also developed an *in vitro* co-culture model with stromal cells and osteoclasts to distinguish the effect of drugs or changes in culture conditions, on myeloma cells from other co-cultured cells.

5.3 Characterisation of eGFP expressing MM1S and MM1R MM cell lines

Infection with lentiviral vectors had 97% and 80% efficiency in transducing MM1S and MM1R cell lines respectively, with the lentivirus DNA coding for GFP. This was confirmed by measuring GFP expression on flow cytometry (Figure 1middle panels). Following clonal selection by limiting dilution assay as described in materials and methods, 4 clones of eGFP-MM1S cells and 5 clones of eGFP-MM1R cells were selected. The clones were expressing eGFP homogenously in comparison with the initially transduced parental population (Figure 1 right panels), which supports their clonality. The expression of eGFP using lentivirus transduction and the cloning process using limiting dilutions may alter the growth potential of these cell lines. To confirm the growth potential, cell proliferation of 4 clones of eGFP-MM1S and 5 clones of eGFP-MM1R was evaluated over 96 hours using an MTT assay. Cells were plated in parallel with the parental cell lines (Figure 2, 3). There were no major differences in the pattern of proliferation with respect to parental cell lines by MTT assay. Growth kinetics of eGFP-MM1S Clone 3 and MM1R Clone 2 GFP were almost identical to the parental cell lines and were used in all further experiments described in this thesis. Dual peaks of eGFP expression were observed by flow cytometry primarily in clonal cultures of eGFP expressing MM1S cells during the initial days of proliferation before entering exponential growth. It was also observed that the lower intensity eGFP signal corresponded to cells with smaller forward scatter and increased side scatter (Fig 4). Previous experiments with parental cell lines described in this thesis showed that an increase in this subpopulation of cells in non-proliferative cultures which may correspond to senescent cells or cells

entering apoptosis. Hence, I investigated whether these changes in eGFP levels were dependent on the phase of cell cycle or cell viability. Lower eGFP levels were predominantly expressed by eGFP-MM1S cells in the sub-G0 phase of the cell cycle (Fig 5). Treatment of eGFP-MM1S cells with Dexamethasone resulted as expected in an increase in the percentage of cells in the sub-G0 population. This sub-G0 population corresponded to the population of eGFP-MM1S expressing low levels of eGFP. A proportion of low eGFP expressing cells were also in the G0 phase (non – proliferating fraction). This confirms that the generated eGFP-MM1S cells are clonal and the observed population of low-expressing eGFP cells, which also display lower forward scatter and higher side scatter in the initial and final stages of the cultures correspond to non proliferating (G0) or predominantly to apoptotic cells (sub-G0 population).

Figure 1. eGFP transduction and clonal selection. eGFP levels detected by flow cytometry in MM1S (upper panels) and MM1R cells (lower panels) pre and post GFP transduction and after clonal selection by limiting dilution.

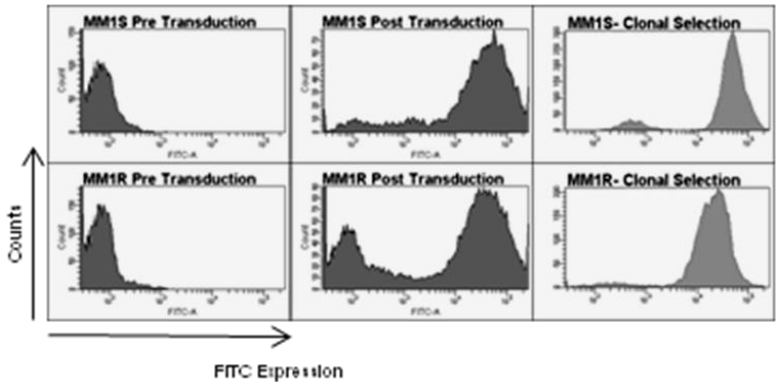


Figure 2: Growth curve of parental and eGFP MM1S clones. Growth curve of MM1S, MM1S GFP clones 1 -4 analysed by MTT assay over 96 hours. Parental and eGFP-expressing cells proliferated at the same rate since we found no statistical differences between the rate of increase in number of cells per day (Student's t-test)

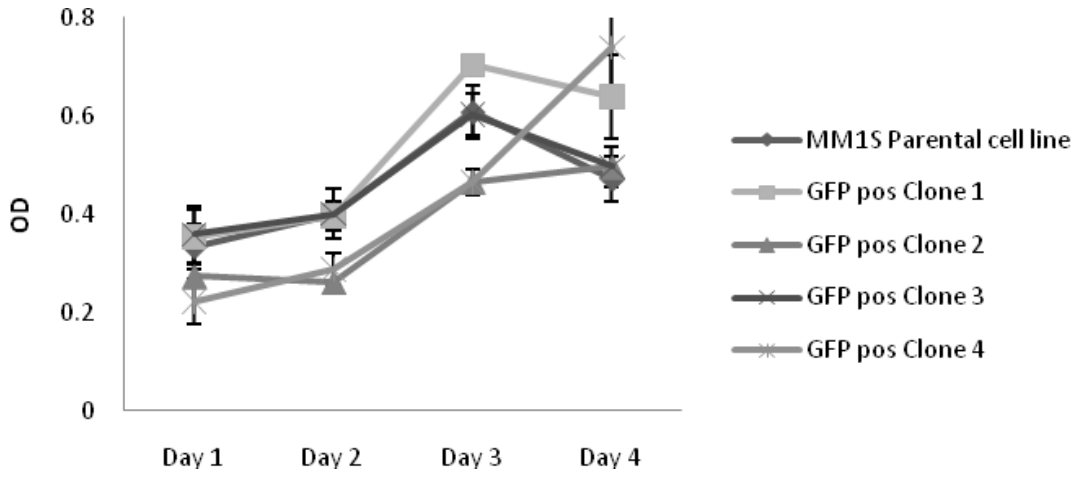


Figure 3: Growth curve of parental and eGFP MM1R clones. Growth curve of MM1R and MM1R GFP clones 1-5 analysed by MTT assay over 96 hours. Parental and eGFP-expressing cells proliferated at the same rate since we found no statistical differences in the number of cells per day (Student's t-test)

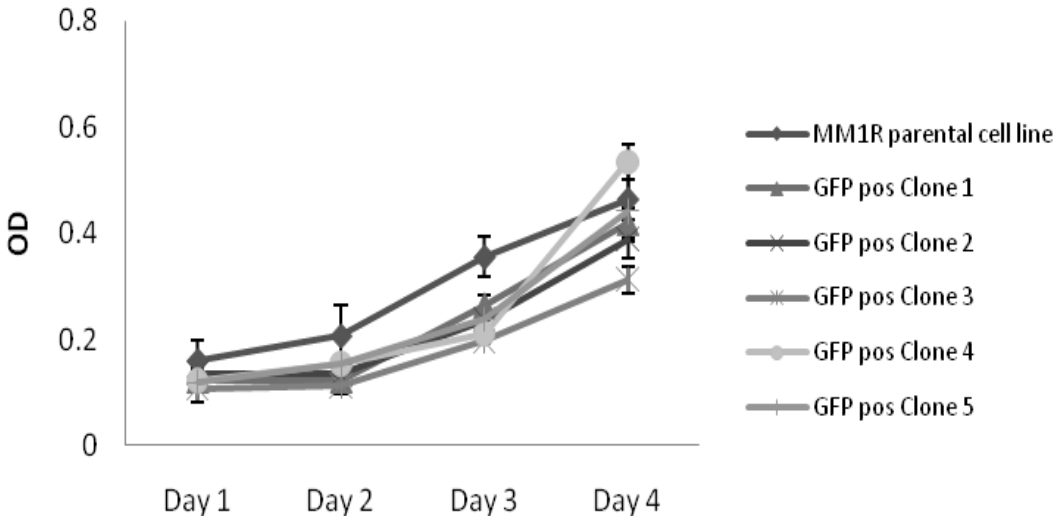


Figure 4: Distinct populations of eGFP expressing cells. (A) Dot plot of forward and side scatter as detected by flow cytometry in MM1S cells left untreated showing two populations of cells (grey and black); (B) Histogram showing levels of expression on eGFP in MM1S cells. Data show that the population with lower forward scatter and larger side scatter expressed lower levels of eGFP (Gate P2) as seen on histogram B.

A **B**

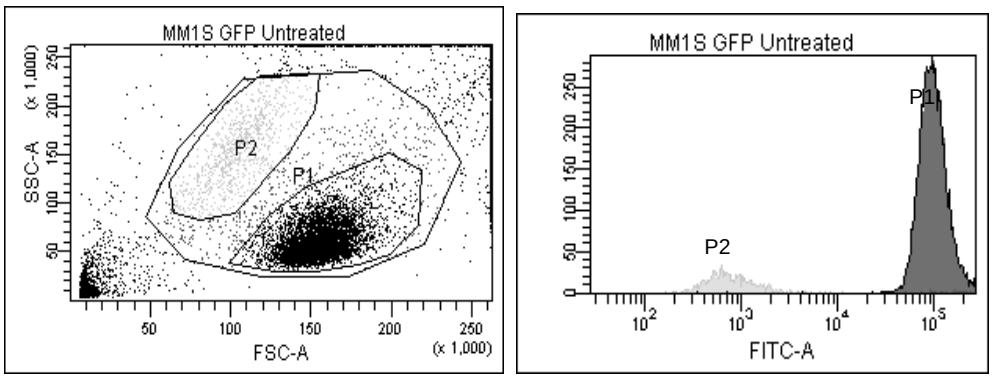
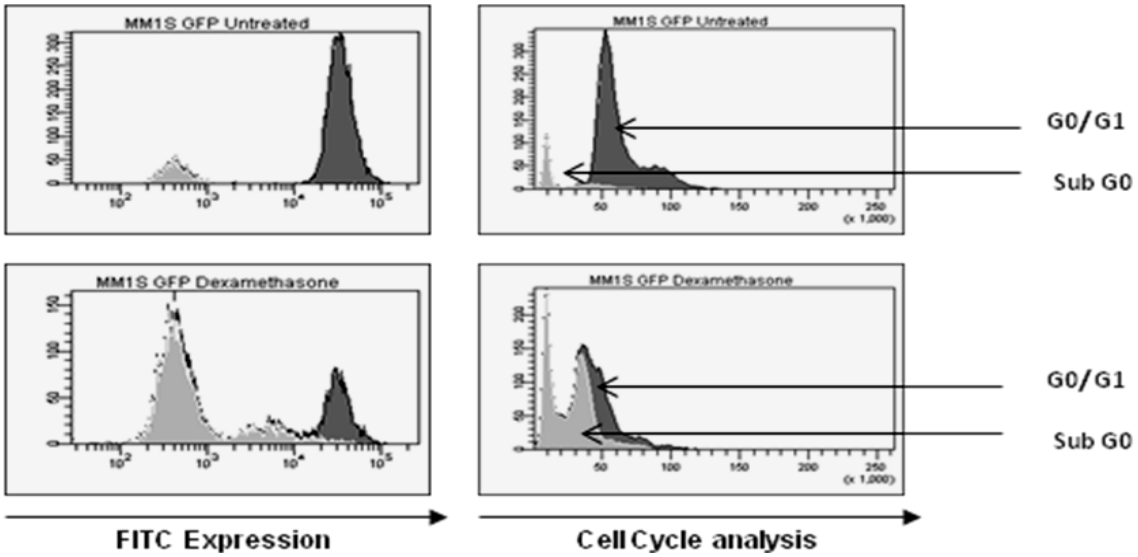


Figure 5: Cell cycle analysis of eGFP expressing cells. MM1S cells expressing eGFP were either left untreated or treated with dexamethasone for 48 hours. Cells were fixed, permeabilised and stained with propidium iodide and cell cycle analysis was performed. Cells expressing lower levels of eGFP corresponded to cells in G0 or sub G0 of the cell cycle.



5.4 Cross validation of use of fluorimetry for enumerating cell numbers and cell proliferation

MTT assays are colorimetric assays which are based on mitochondrial activity of cells. They are widely used for cell number estimation by most laboratories. The intensity of fluorescence from the eGFP-MM cell lines can be measured as fluorescence intensity (FI) in arbitrary units using a fluorescent plate reader. To establish if measuring FI emitted by MM cells was a sensitive method to estimate the number of cells in culture per well, with similar threshold of detection as commonly used methods such as MTT assay, I compared the sensitivity of detection of cell numbers by measuring FI before obtaining OD signal (MTT assay) in identical wells. eGFP-MM1S and eGFP-MM1R cells were plated at 1, 5, 10, 25 and 50 x 10^4 per well which corresponds to a maximum density of 2.5 x 10^6 cells per ml. Both the OD signal and the FI measurement of eGFP-MM cell lines were equally proportional to the number of cells plated per well (Figure 6). There was good correlation between MTT assays and Fluorescence detection in both MM1S GFP and eGFP-MM1R cells with a coefficient of determination R_2 = 0.99 (Figure 7). This data supports the use of fluorescence detection by a fluorescence plate reader as a reliable substitute for cell number estimation in the place of colorimetric MTT assays, in GFP expressing cells.

MM1S and MM1R cell lines differ in their sensitivity to Dexamethasone treatment. MM1R cells are resistant to Dexamethasone due to presence of truncated glucocorticoid receptor[158,182]. To test the experimental use of fluorimetry in comparison to standard MTT assay and to confirm that the eGFP-expressing cells retained the characteristics of the parental non-fluorescent cells, we studied the effect of Dexamethasone treatment on the cultures. Cells were left either untreated

or treated with Dexamethasone for 4 days and both fluorimetry and spectrophotometry were performed to compare the methods. The eGFP-MM1S cells continued to be sensitive to Dexamethasone and eGFP-MM1R cells were resistant retaining the parental cell characteristics (Fig 8 A). The results obtained by fluorimetry were equivalent to the commonly used spectrophotometric detection (MTT assay) (Figure 8 B). Taken together the results show that measuring fluorescence intensity of eGFP-MM1S/MM1R cells is a valid method to estimate the numbers of cells in culture with the same sensitivity as standard methods such as the MTT assay.

Figure 6: Comparison between fluorimetry and MTT assay. MM1S GFP (A, C) and MM1R GFP (B, D) were plated at 1, 5, 10, 25, 50 x 10^4 cells/ well in a 96 well plate and both fluorimetry, fluorescence emitted per well eGFP in arbitrary units (AU) (A, B) and MTT assay, optical density (OD) per well (C, D) were measured.

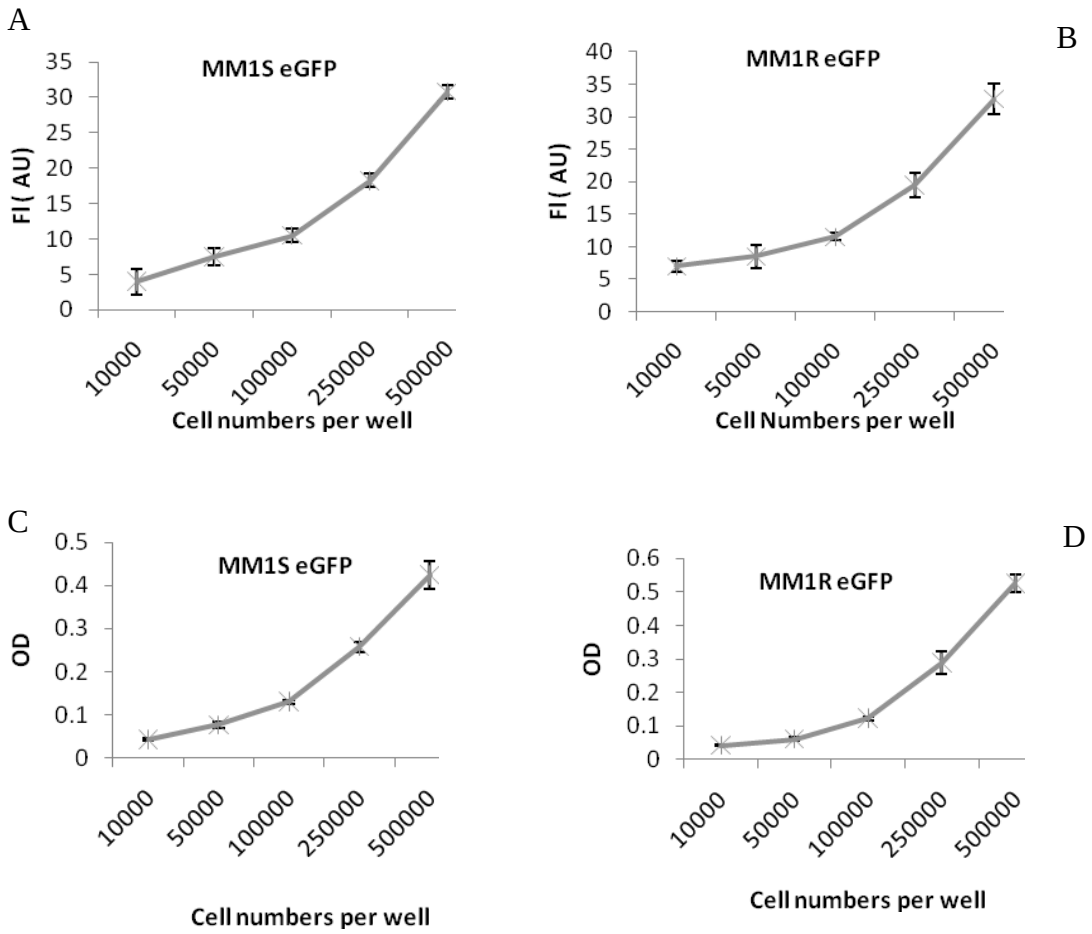
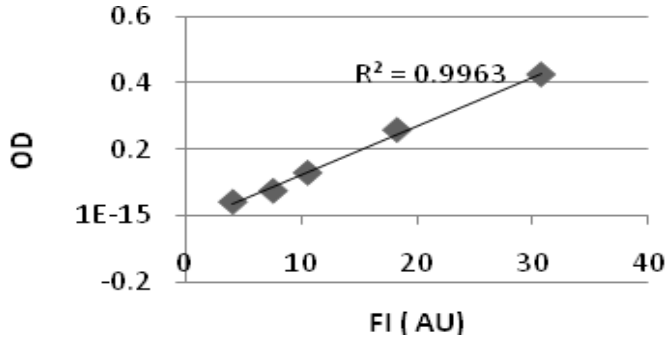


Figure 7: Correlation curve. Correlation between OD and FI in MM1S GFP (A) and MM1R GFP cells (B) using linear regression. R2 – coefficient of determination

A



B

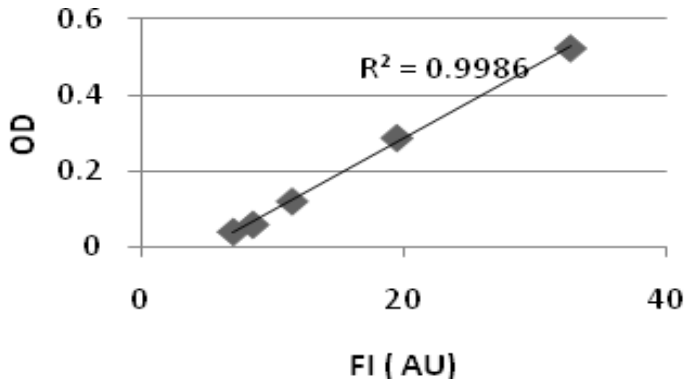
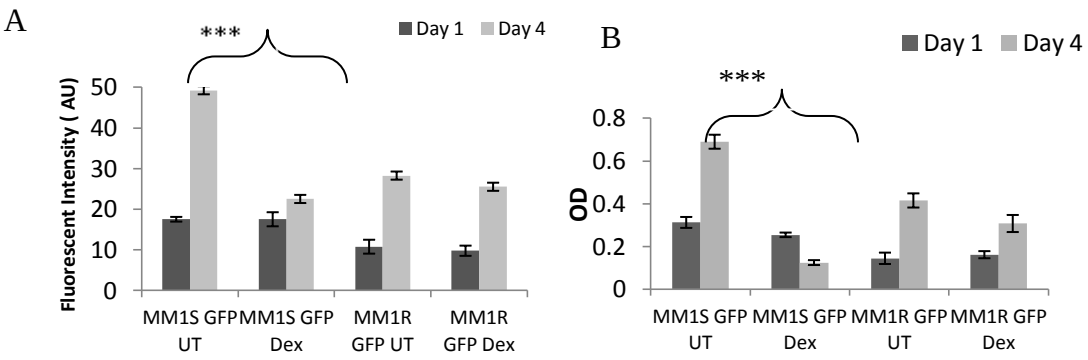


Figure 8: Drug sensitivity testing by flourimetry and MTT assay. MM1S and MM1R GFP cells left untreated or treated with Dexamethasone over 4 days and proliferation assessed by fluorimetry (A) and MTT assay (B). Differences in cell growth were statistically analysed by comparing the growth index with respect to the number of cells at day 0 (Student's t-test. *** p, 0.005)



5.5: eGFP expressing MM cell proliferation and viability in the presence of BM stromal cells

MTT assay is commonly used when testing sensitivity of agents on co-culture of plasma cells and stromal cells [179]. But this method is fraught with problems as the signal generated is from mitochondrial activity of both cell types and it is not possible to differentiate between changes in cell numbers of plasma cells or the other cells in co-culture. In certain cases, the effect of certain drugs could have differential effects on the accessory cells in coculture with the plasma cell giving erroneous results. As shown in the previous section, the fluorescent signal from eGFP-MM1S and eGFP-MM1R cells correlates with cell numbers in culture. Hence, if co-cultured with other non-fluorescent cells, we could record the emission of fluorescence intensity over time and discern the growth of eGFP-MM cell lines without interference of emission from the other cell types in co-culture.

For cell proliferation assays of MM cell lines in co-culture, Human HS5 stromal cells (SC) were plated at a density of 0.3 x 10^5 cells per well in a 96 well plate overnight. Media was aspirated and eGFP positive MM cell lines were plated at a density of 4 x 10^5 cell/ml (eGFP-MM1S) and 2 x 10^5 cell/ml (eGFP-MM1R) on the semi-confluent SCs. On the days of assessment, plates were read in FLx800 multidetection plate reader as described in materials and methods. For FACS assays, HS5 SCs were plated at a density of 10^5 cell/ well overnight in a 24 well plate. GFP positive plasma cells were added at the above density with and without treatments. On the days of assessment, suspended cells (which corresponded mainly to PC) were collected and wells washed with PBS once to retrieve the remaining PC which were loosely

adhered on SCs. We confirmed by flow cytometry that the remaining cells in the well were eGFP negative corresponding to SCs only (Figure 9).

Using this method, we compared the effect of Dexamethasone on eGFP-MM1S and eGFP-MM1R cells cultured alone or in the presence of the HS5 SC. Co-culture of eGFP-MM1S cells with SCs blocked the anti-proliferative effect of Dexamethasone and MM plasma cells continued to proliferate (Figure 10 A). As expected, eGFP-MM1R cells cultured alone were resistant to Dexamethasone treatment and continued to proliferate in the presence of the drug independent of the presence of stromal cells (Figure 10 B). The proliferation data in the eGFP cell lines correlated with the levels of apoptosis detected by expression of Annexin V in plasma cells detected by FACS in the eGFP positive MM population. Treatment of eGFP-MM1S alone with 2 μM Dexamethasone resulted in extensive apoptosis with 95% of eGFP-MM1S cells expressing Annexin V (Figure 11 A). However, when cells were co-cultured with stromal cells, we detected that 60 % of eGFP-MM1S remained viable (Figure 11). As expected, eGFP-MM1R cells remained viable in the presence of Dexamethasone with very low levels of apoptosis independent of the presence of stromal cells (Figure 11 B). Taken together, our proliferation and apoptosis data indicate that stromal cells provide a protective environment for eGFP-MM1S cells, which would normally be sensitive to treatment with Dexamethasone. Our results also show confirmed that the genetic pool of eGFP-MM1R cells [158] make them resistant to Dexamethasone regardless of the presence of SCs.

We also found that eGFP-MM1S cells failed to proliferate when cultured in serum free medium to remove all signals from soluble factors, whereas eGFP-

MM1R cells continued to proliferate (Figure 10) and remained largely viable in the absence of serum with only 25% of cells undergoing apoptosis, whereas 87% of apoptotic cells were detected in eGFP-MM1S cultures in the absence of serum (Figure 11). Co-culture of eGFP-MM1R cells with stromal cells in serum free medium modestly promoted viability and proliferation of eGFP-MM1R but not eGFP-MM1S cells (Figure 11). Taken together, our results indicate that eGFP-MM1R cells can induce autocrine stimulation leading to survival possibly by release of soluble factors. There is also a suggestion that eGFP-MM1R but not eGFP-MM1S cells have a higher potential of interaction with SCs leading to MM cell proliferation, in the absence of soluble factors from the serum.

Fig 9: Flow cytometric determination of ability of extracting plasma cells from PC-SC cocultures. MM1S GFP and MM1R GFP cells were plated on HS5 stromal cells (SC) or grown alone in 96 well plate for 4 days either left untreated (UT), treated with Dexamethasone (Dex) or cultured in serum free medium (SF). After 4 days eGFP plasma cells were collected and stromal cells remaining in the well were assayed to look for plasma cell contamination (FITC positive).

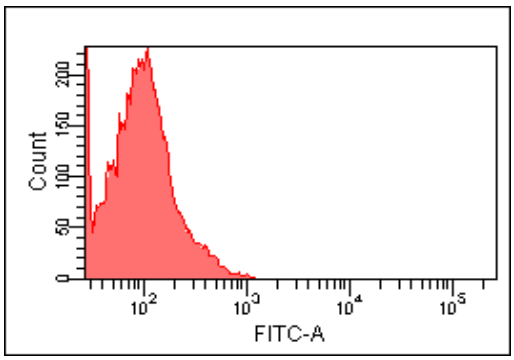
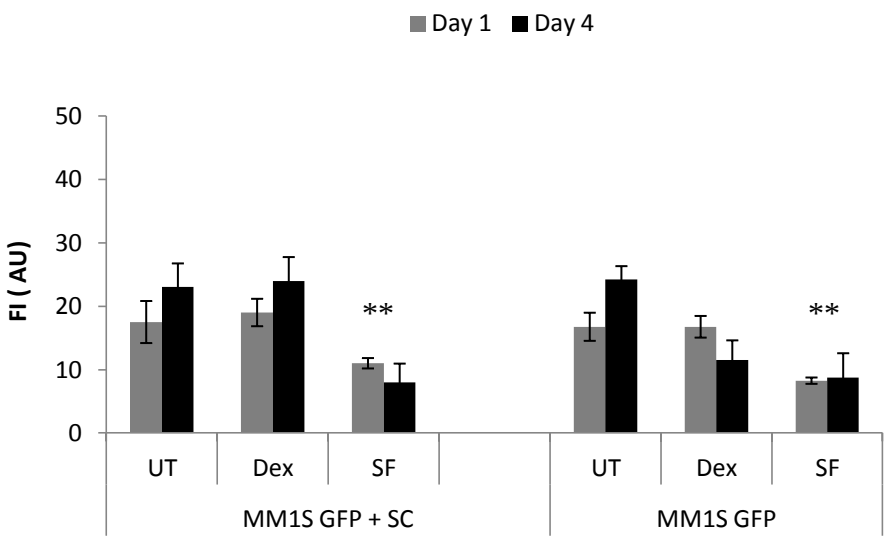


Figure 10. Evaluation of proliferation and viability in eGFP expressing MM cells alone or in co-culture with human stromal cells. (A, B) MM1S GFP and MM1R GFP cells were plated on HS5 stromal cells (SC) or grown alone in 96 well plate for 4 days either left untreated (UT), treated with Dexamethasone (Dex) or cultured in serum free medium (SF) and fluorescence emission per well was determined using a fluorescence plate reader. Differences in cell growth were statistically analysed by comparing the growth index with respect to the number of cells at day 0 (Student's t-test. ** p< 0.005, # p, 0.05).

A


Day 1
Day 4
FI (AU)
50
40
30
20
10
0
**
**
UT
Dex
SF
UT
Dex
SF
MM1S GFP + SC
MM1S GFP

B

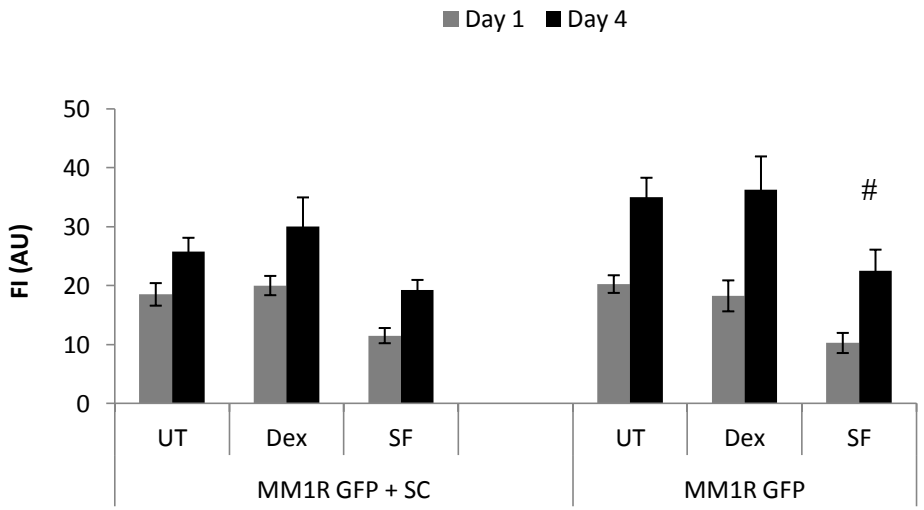
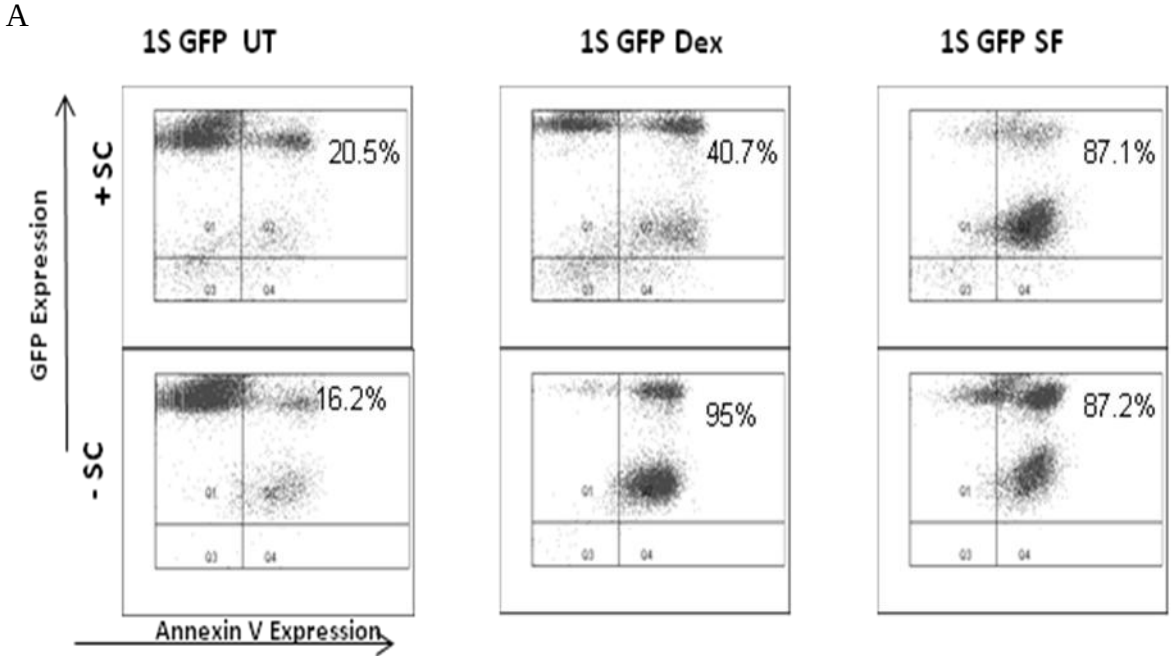

Day 1
Day 4
FI (AU)
50
40
30
20
10
0
#
UT
Dex
SF
UT
Dex
SF
MM1R GFP + SC
MM1R GFP

Figure 11: Flow cytometric evaluation of apoptotic fraction in eGFP positive cells in cocultures with stromal cells with and without treatment. Annexin V expression of MM1S (A) and MM1R GFP cells (B) plated on HS5 stromal cells or cultured alone for 4 days determined by flow cytometry. Flow cytometry scatter plots show expression of eGFP in the Y axis and expression of Annexin V-APC in the X axis of the eGFP positive population in the co-cultures.

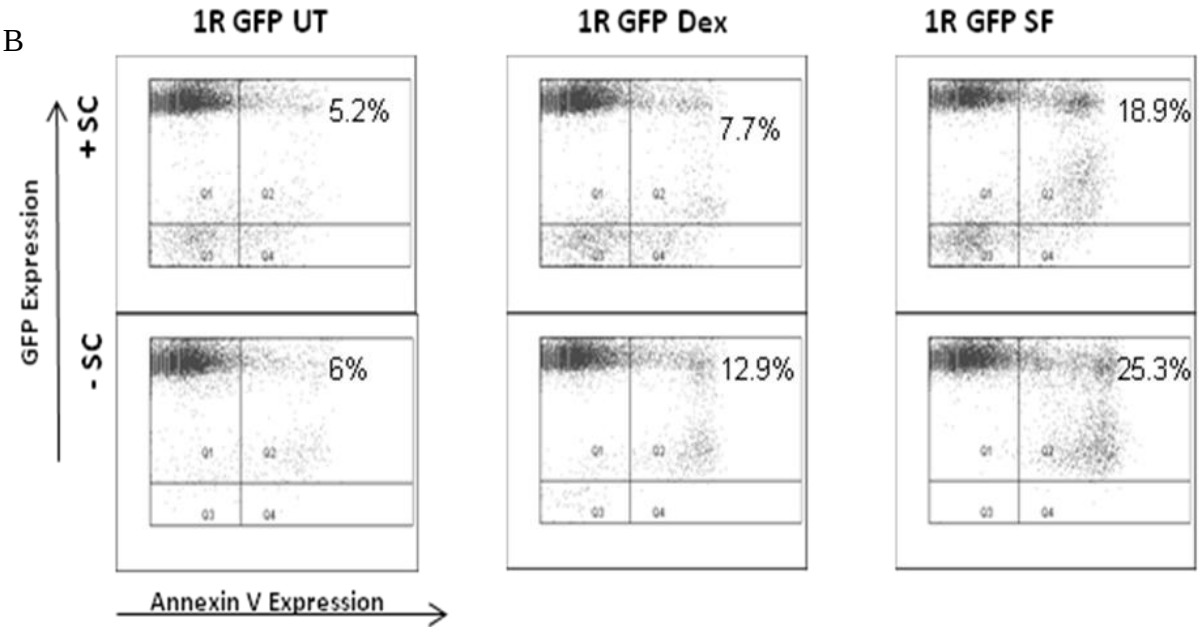
MM1S

A


1S GFP UT
1S GFP Dex
1S GFP SF
GFP Expression
+ SC
- SC
20.5%
16.2%
40.7%
95%
87.1%
87.2%
Annexin V Expression

MM1R

B


1R GFP UT
1R GFP Dex
1R GFP SF
GFP Expression
+ SC
- SC
5.2%
6%
7.7%
12.9%
18.9%
25.3%
Annexin V Expression

5.6 Proliferation and viability in eGFP expressing MM cells alone or in co-culture with osteoclasts.

Osteoclasts play a key role in proliferation and survival of myeloma cells. Osteoclasts are activated in the microenvironment of myeloma patients and hence the effect of therapeutic interventions should be studied in the presence of osteoclasts. These have been detailed earlier in section 2.6.1. The key problems with using an MTT assay in cocultures have been discussed in the last section. Anderson et al studied effects of myeloma drugs on osteoclastogenesis and 2 key problems were observed when the experiments were performed. Cell doublets are formed in osteoclast cultures and they have to be gated out during analysis of FACS plots. The drug treated wells demonstrate mononuclear cells which are arrested in pre lineage commitment phase and therefore use of cell size or expression of surface proteins for gating in or out can be challenging. Clone hybrid cells are generated in osteoclast- plasma cell cocultures. These cells are thought to be resultant of a fusion between myeloma cells and osteoclasts[183]. These experimental observations underpin the importance of developing a myeloma coculture system to study the effects on plasma cells.

Osteoclasts were differentiated over 3 weeks from primary BM mononuclear cells obtained from MM patients as previously described[44]. GFP positive plasma cells were layered on fully grown osteoclasts in 96 well plates. On the days of assessment, images were obtained using a fluorescence microscope and fluorimetry performed using a multi detection plate reader FLx800. For flow cytometry analysis, GFP positive PC were plated on fully grown osteoclasts in 24 well plates and supernatant containing PC was collected on

the days of assessment. Wells were washed with PBS once to collect the remaining plasma cells. We confirmed by flow cytometry that the remaining cells in the well were eGFP negative; corresponding to osteoclasts only prior to determining apoptotic fraction in the eGFP positive plasma cells (Figure 12).

eGFP-MM1S cells treated with Dexamethasone alone or in co-culture with osteoclasts failed to proliferate as detected by fluorimetry (Fig 13 A). As expected, eGFP-MM1R cells were able to proliferate and were resistant to Dexamethasone independent of the presence of osteoclasts (Figure 13 B). . Although treatment with Dexamethasone inhibited proliferation of eGFP-MM1S cells despite the presence of osteoclasts, using flow cytometry we observed that osteoclasts significantly reduced the pro-apoptotic effect of Dexamethasone on eGFP-MM1S cells (Figure 14 A). Additionally, osteoclasts protected eGFP-MM1S and eGFP-MM1R cells from serum starvation as observed by decreased Annexin V expression in PC co-cultured with osteoclasts compared to PC grown alone (Fig 14). This supports data previously published [49,69]. However, neither eGFP-MM1S nor eGFP-MM1R cells proliferated in co-culture with osteoclasts in serum-free medium (Fig 12). This data confirms that osteoclasts provide prosurvival effects in serum free conditions by direct cell-cell contact for both dexamethasone sensitive and resistant cells.

Fig 12: Flow cytometric determination of ability of extracting plasma cells from PC-SC cocultures. MM1S GFP and MM1R GFP cells were plated on osteoclasts (OC) or grown alone in 96 well plate for 4 days either left untreated (UT), treated with Dexamethasone (Dex) or cultured in serum free medium (SF). After 4 days eGFP expressing plasma cells were extracted and osteoclasts were assayed to look for plasma cell contamination (FITC positive).

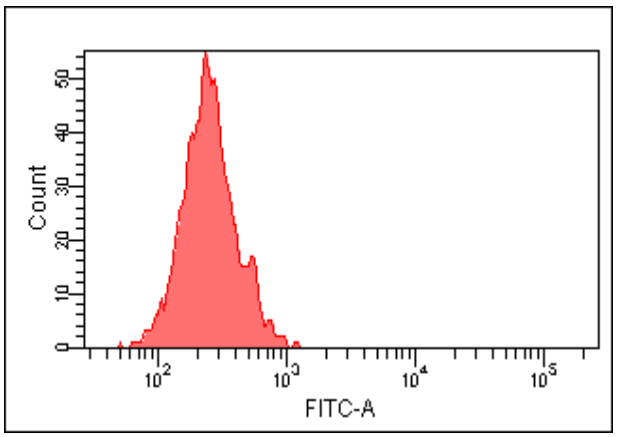
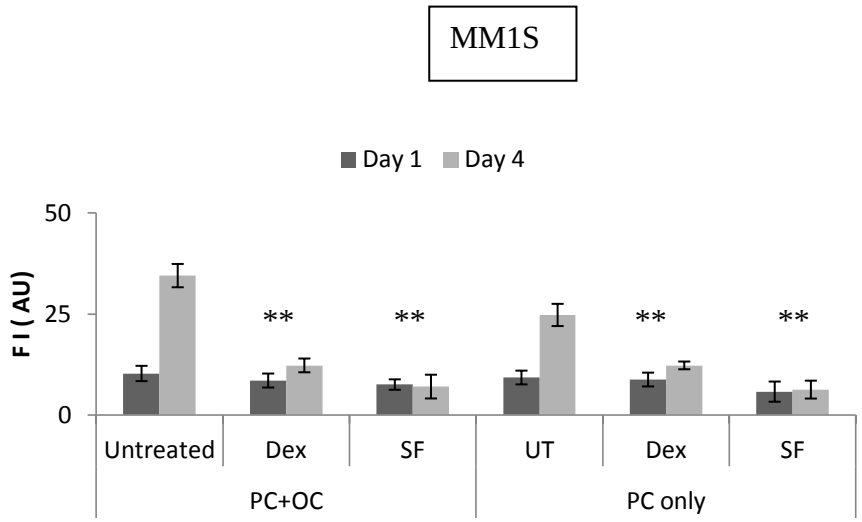


Figure 13: Evaluation of proliferation and viability in eGFP expressing MM cells alone or in co-culture with osteoclasts. (A, B) MM1S GFP and MM1R GFP cells were plated on osteoclasts (OC) or grown alone in 96 well plate for 4 days either left untreated (UT), treated with Dexamethasone (Dex) or cultured in serum free medium (SF) and fluorescence emission per well was determined using a fluorescence plate reader. **P<0.005 student t-test.

A



B

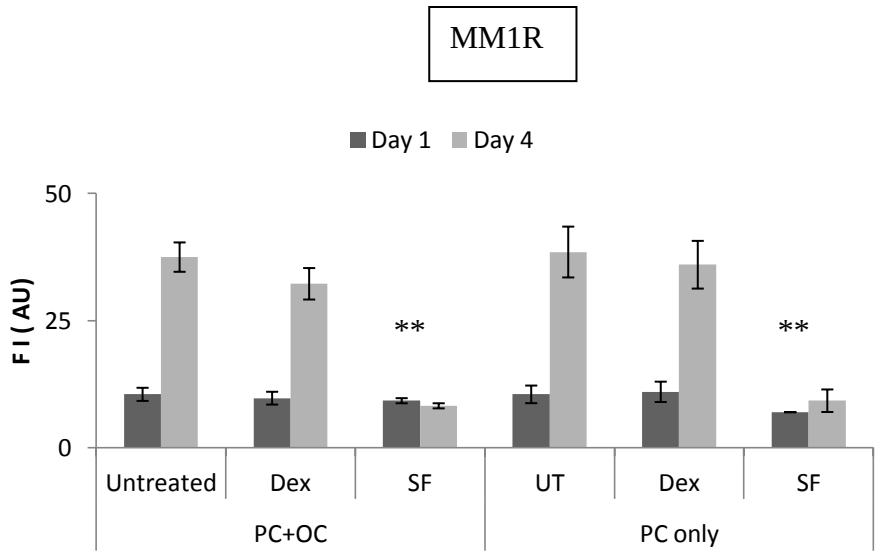
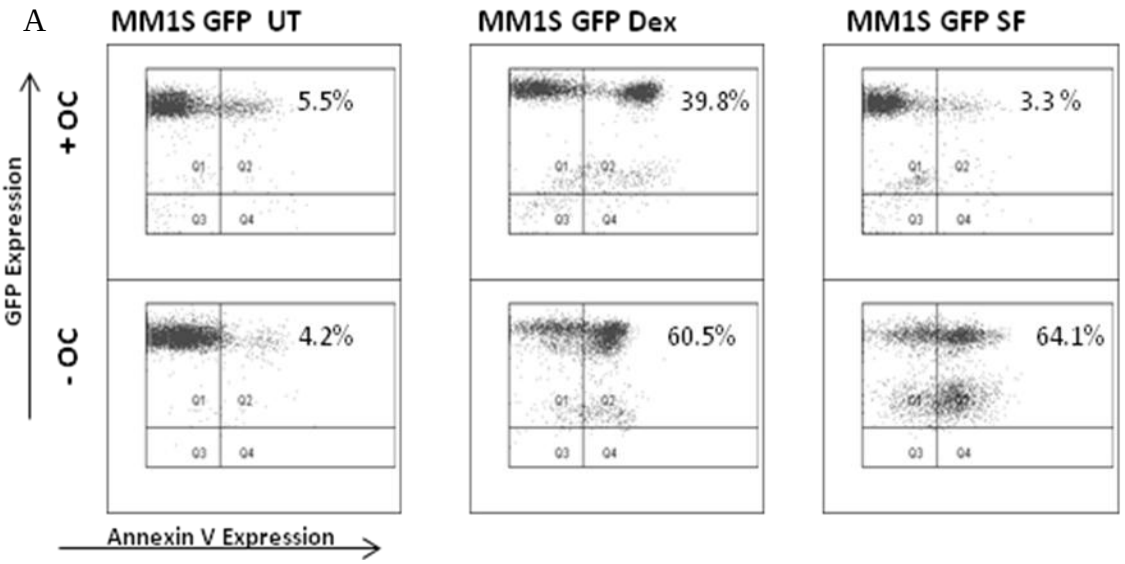
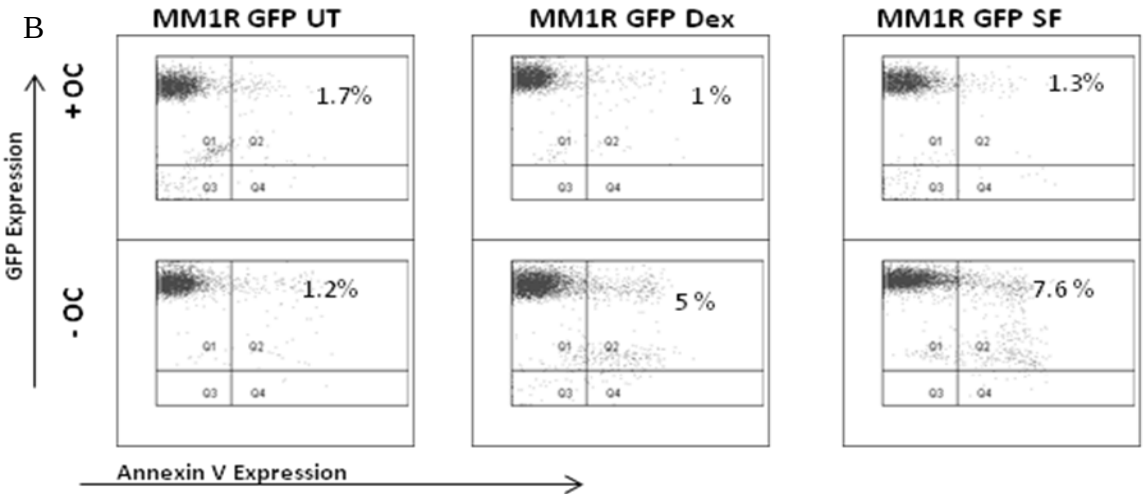


Figure 14: Flow cytometric evaluation of apoptotic fraction in eGFP positive cells in cocultures with osteoclasts with and without treatment. MM1S GFP and MM1R GFP cells were plated on osteoclasts (OC) or grown alone in 96 well plate for 4 days either left untreated (UT), treated with Dexamethasone (Dex) or cultured in serum free medium (SF) Annexin V expression of MM1S (A) and MM1R GFP cells (B) plated on osteoclasts or cultured alone for 4 days determined by flow cytometry. Scatter plots show expression of eGFP in the Y axis and expression of Annexin V-APC in the X axis of the eGFP positive population in the co-cultures.

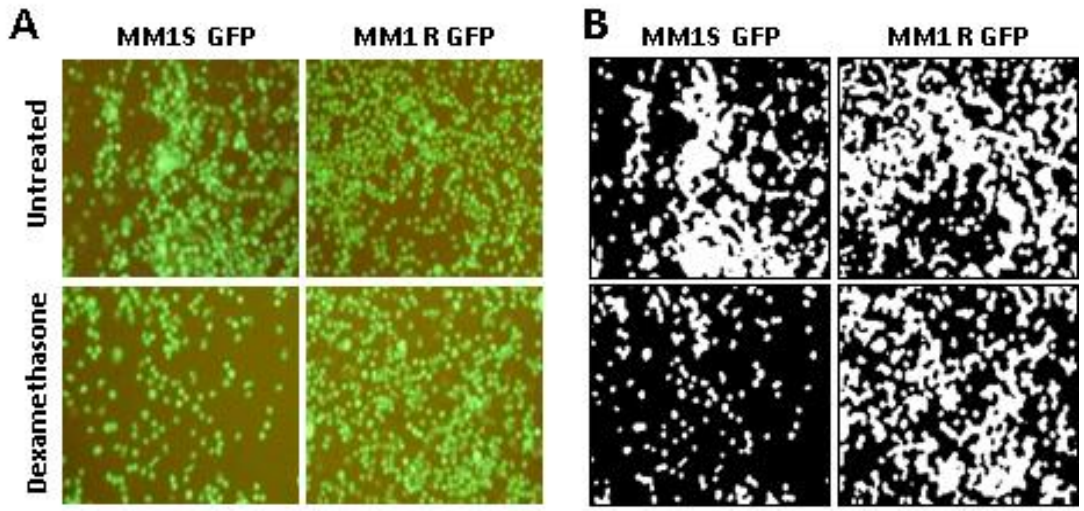

A
MM1S GFP UT
MM1S GFP Dex
MM1S GFP SF
+ OC
- OC
GFP Expression
5.5%
39.8%
3.3 %
4.2%
60.5%
64.1%
Q1
Q2
Q3
Q4
Annexin V Expression

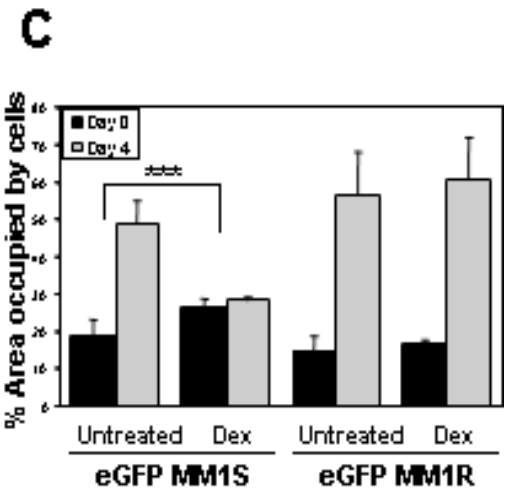

B
MM1R GFP UT
MM1R GFP Dex
MM1R GFP SF
+ OC
- OC
GFP Expression
1.7%
1 %
1.3%
1.2%
5 %
7.6 %
Q1
Q2
Q3
Q4
Annexin V Expression

5.7 Image-based analysis of eGFP-MM cells proliferation

Using eGFP-MM cells, proliferation can also be determined by image analysis. Images of cultured cells can be obtained using a fluorescent microscope (Fig 15 A) and quantification of the area occupied by these cells can be performed similarly as previously described [184]. Images of eGFP cells were captured in co-culture with osteoclasts (Figure 15). We processed the obtained images using Photoshop to generate a black and white image as described in materials and methods (Figure 15 B). Image Tool software was used to quantify the percentage of white pixels per image, which corresponded to the area occupied by MM cells (Figure 15 C). Our data show that by estimating the area occupied by MM cells (Figure 15C), we can determine proliferation with comparable sensitivity to fluorimetry (Figure 12 A, B). Hence, both methods (measurement of FI using a plate reader or image analysis of micrographs) can be used to determine the number of eGFP-MM cells per well in co-culture with other cells of the BM microenvironment.

Figure 15: Determination of cell proliferation using image analysis of micrographs eGFP-MM cell. eGFP-MM1S and eGFP-MM1R cells were plated in co-culture with osteoclasts and left untreated or treated with 2 μM Dexamethasone (Dex). Image processing and quantification of micrographs of eGFP-MM cells in co-culture with osteoclasts (A) Micrographs of eGFP cells in co-culture with osteoclasts. Images only show the presence of MM cells with no interference of the other cells in co-culture. Bar 50 μm (B) Binary images were obtained from the original fluorescent micrographs after processing using Adobe Photoshop CS4 as described in materials and methods. (C) Using UTHSCSA Image Tool processing software we quantified the percentage of white pixels per micrograph, which correspond to the percentage of the area of the well occupied by eGFP-MM cells (n = 10 fields for group of analysis). Differences in cell growth were statistically analysed by comparing the growth index with respect to the area occupied by cells at day 0 (Student's t-test. *** p,0.005). Our results show that measuring the percentage of the area occupied by eGFP-MM1S cells using image analysis is as effective to determine cell numbers in culture as measuring fluorescence per well using a fluorescence plate reader.


A
MM1S GFP
MM1 R GFP
Untreated
Dexamethasone
B
MM1S GFP
MM1 R GFP


C
% Area occupied by cells
Day 0
Day 4
****
Untreated
Dex
Untreated
Dex
eGFP MM1S
eGFP MM1R

5.9 Discussion

MM remains incurable partly because of presumed permissive cellular microenvironmental niches and the presence of soluble factors and extracellular matrix proteins that create a BM milieu that renders relative resistance to myeloma cells against therapeutic agents. A great amount of evidence supports a key role of the tumour microenvironment in sustaining survival and proliferation of MM plasma cells within the BM [54]. It is plausible that the in-vivo effect of any drug would be only partially captured by treatment of MM cells and BM cells separately. The impact of drug treatments should therefore be studied under experimental conditions that bear the closest similarity to that of the real tumour microenvironment. This requires the development of new co-culture systems to allow discrimination of the effects of drugs on the MM cells from that on other BM cells. Several groups have already developed strategies to recreate a tumour microenvironment *in vitro* to study anti myeloma efficacy of drugs [180,185] but their settings are laborious and technically challenging. Additionally, commonly used *in vitro* assays are not applicable in co-culture settings to discriminate the effect of drugs on MM cells from the rest of BM cells in co-culture as the detected signal is produced by all cells in the culture. Recent work by McMillin et al proposed the use of tumour cells stably expressing luciferase to address these issues and showed they can analyse and distinguish the effect of therapeutic drugs on viability and proliferation of tumour cells in co-culture with SC [181]. Our work shows that expression of eGFP in MM cell lines equally allows analysis of proliferation and viability of MM cells in co-culture with other BM cells. Compared to the previously described method using bioluminescence, the use of fluorescent

cells is much simpler and less expensive because substrate is not required and the fluorescence emitted per well by the eGFP in MM cells is read directly in a fluorescence plate reader. Expression of eGFP in the two MM cell lines tested did not interfere with their normal rate of proliferation or their intrinsic properties such as sensitivity to Dexamethasone. Our data expand the applicability of eGFP-MM cells so that they can be used for proliferation assays in co-culture with other BM cells as well as *in vivo* for previously described applications such as tracking of engraftment of MM cells in mice [95,158] or for further gene expression profiling after FACS sorting [181]. Detection of apoptotic MM cells in co-culture with other BM cells by flow cytometry is currently based on harvesting of co-cultured cells and staining with anti-CD138 antibodies to detect the presence of plasma cells and anti-Annexin V to detect MM apoptotic cells. However, CD138 expression in MM plasma cells can be lost in long term co-cultures with osteoclasts[186]. We propose the use of eGFP-expressing MM cell lines as a more consistent method to measure the levels of MM cell death by apoptosis in co-culture with other BM cells. By harvesting co-cultures and using FACS, we can reliably detect apoptotic cells (Annexin V positive) in eGFP expressing cells (MM cells). The combination of the data obtained using fluorimetry (cell proliferation) and FACS (cell apoptosis) are essential to understand the real effect of the microenvironment or drugs on MM cells in co-culture.

Using our method, we have shown that in the absence of soluble factors provided by foetal bovine serum used in *in vitro* culture, both eGFP-MM1R (resistant to Dexamethasone) and eGFP-MM1S cells (sensitive to Dexamethasone) failed to proliferate as shown using fluorimetry. However,

using flow cytometry, we were able to show that a high percentage of eGFP-MM1R cells remained viable in serum-free medium whereas eGFP-MM1S cells apoptosed in the absence of serum. When co-cultured with SC in serum-free medium, MM1S cells in the presence or absence of SC failed to proliferate and underwent apoptosis, whereas SC promoted eGFP-MM1R cells proliferation. However, when co-cultured with osteoclasts in serum-free medium, although viability of both cell types increased as shown before [69], neither eGFP-MM1S nor eGFP-MM1R cells were able to proliferate. Taken together our results show that eGFP-MM1R cells are able to survive independent of the presence of the BM microenvironment suggesting that these cells are able to secrete or favour the pathway of pro-survival factors, which may account for their acquired Dexamethasone-resistant phenotype. This is supported by previous characterisation of MM1S and MM1R cell lines, which showed that various pro-survival signalling proteins, transcription factors and receptors for transcription factors are up regulated in MM1R cells [158]. Additionally, in the absence of serum, MM1R but not MM1S cells proliferated in co-culture with SC. This suggests that MM1R cells can induce or respond to SC factors more efficiently than MM1S cells. For example, MM1R have the potential to more readily respond to SC-secreted IL-6 since the IL-6 pathway is enhanced by upregulation of IL-6 signal transducer gp130 [158]. This model is ideal to precisely understand the mechanisms of resistance to therapeutic drugs as we are able to study various aspects of eGFP-MM cells in co-culture with other cells in the BM microenvironment.

Our results confirmed as expected that Dexamethasone treated eGFP-MM1R cells proliferated and remained viable independent of the presence of SC or

osteoclasts. In the case of eGFP-MM1S cells, both SC and osteoclasts promoted eGFP-MM1S cells viability but only SC sustained eGFP-MM1S proliferation. These results suggest that fibroblastic SCs promote MM cell proliferation more efficiently than OCs. Taken together, these results suggest that eGFP-MM1R cells can secrete autocrine pro-survival factors that may account for their Dexamethasone-resistant phenotype independently of the tumour microenvironment. Additionally, although eGFP-MM1S cells are sensitive to treatment with Dexamethasone on their own, they are able to resist the treatment with Dexamethasone through the protection provided by osteoclasts and SC. Our data suggest that the microenvironment provided by SC or osteoclasts in the BM promotes resistance to Dexamethasone *in vivo* to MM plasma cells that otherwise would be sensitive to treatment as they lack a resistant phenotype supported by their own gene expression. It is likely that adhesion of eGFP-MM cells may promote secretion of soluble factors like IL-6 by SC [187] or osteoclasts [69], which not only blocks Dexamethasone-induced apoptosis but also promotes MM cell proliferation [188,189].

These experiments demonstrate the potential use of eGFP-MM cell lines to increase our understanding of MM tumour biology. Stromal cells derived from long term cultures of CD138 negative fraction from BMs of MM patients can be used in the place of stromal cell line described in these experiments [181,190]. Like in the case of the recently described luciferase-based experimental platform [181], the advantage of our system with respect to other previously taken approaches is that it allows us to discriminate the effect of drugs on MM cells from the effect on other BM microenvironment cells. Since detection of eGFP signal in eGFP-MM cells allow determination of viable cell numbers,

detection using a fluorescent plate reader would also allow determining the adhesion efficiency of eGFP-MM on different substrata including the surface of BM cells. Given that cells can be cultured in multiwell plates, our method is also useful for high throughput screening of drugs and drug combinations against myeloma in the presence of the BM microenvironment. The use of eGFP-MM cells allows application of additional analysis of various parameters of cell function such as cell cycle using FACS. We have also shown that using image based analysis of eGFP-MM cells in co-culture with other BM cells we can estimate proliferation of MM cells. Using currently available microscopy systems that allow high throughput image-based analysis it will be possible to screen various drugs and drug combinations in a short period of time. The use of eGFP-MM cells would also allow us to track MM cells using live video microscopy, which is essential to further understand the adhesion and migration properties of MM cells in the BM microenvironment.

PLASMA CELL ADHESION

6.1 Introduction

Cell adhesion in neoplasia is gradually gaining more significance and despite well understood mechanisms of cell adhesion, under normal physiological conditions the exact regulation of adhesion in the tumour microenvironment remains largely unknown. Plasma cell adhesion to the microenvironment is one of the key properties by which the disease is maintained before eventually relapsing in almost all patients. Persistence of plasma cells in patients who have aplastic bone marrow following chemotherapy could indicate its location in well preserved niches. Adhesion of plasma cells is regulated through receptors on the cells and ligands in the microenvironment. Plasma cells and the marrow microenvironment are coupled by a complex network of interactions mediated by soluble factors and adhesion molecules. Signalling responses in plasma cells are activated by adhesive interactions, which critically regulate proliferation, migration and survival of these cells. Integrins and syndecan-1/CD138 are the major myeloma receptor systems of extracellular matrix, as well as of surface molecules of stromal cells. CD44 and RHAMM are the chief hyaluronan receptors of multiple myeloma cells. The SDF-1/CXCR4 axis has been shown to mediate BM stromal cell mediated drug resistance and homing of multiple myeloma cells to the bone marrow[65]. The levels of expression and activity of these adhesion molecules are controlled by signalling mechanisms, as well as by extracellular stimuli including growth factors and micro environmental conditions. But the microenvironment of myeloma patients is continually modified over time. Changes within both the cellular and extracellular components of myeloma bone marrow microenvironment are detected over a period of time. This is

critical for maintenance of disease. For example, laminin immunohistochemistry performed on the bone marrow of myeloma patients demonstrated no significant differences between the groups tested. The levels of fibronectin and collagen I were reduced in marrow of patients with high plasma cell percentage. Expression of collagen IV in the marrow of MGUS and MM patients was higher than in the marrow from normal donors[191].

The well studied system of integrin activation upon binding to it ligands collagen or fibronectin leading to cell adhesion has been described in myeloma. The expression of sets of integrin receptors in different stages of disease such as MGUS, solitary plasmacytomas, early myeloma and relapsed myeloma has been studied. There is no conclusive pattern which has emerged in these studies[76,192]. There appears to be a specific patterning in integrin receptors present in plasma cells isolated from various sites such as marrow, blood and tonsil. These studies have to be interpreted in the light of the fact that isolated plasma cells do not survive for longer periods and it is not clear whether expression of integrin receptors vary with inside out signalling or with external stimuli. This is also substantiated by the fact that survival of plasma cells ex vivo is dependent on a combination of external stimuli, some of which are adhesion dependent[193]. Dalton et al have shown adhesion of myeloma cells to fibronectin induces G0/G1 cell cycle arrest and are resistant to doxorubicin[92]. In their recent work they showed presence of IL-6 although enhances plasma cell proliferation but they do not reverse doxorubicin resistance. Also presence of IL-6 enhanced STAT3 phosphorylation and recruitment of gp130[194].

Bone marrow stromal cells produce and incorporate several types of glycosaminoglycans, which are incorporated in the extracellular matrix. Glycosaminoglycans are part of the molecular structure of proteoglycans such as heparan sulphate, chondroitin sulphate and dermatan sulphate, as well as hyaluronan. Adhesion on stromal cells provides survival benefit for myeloma cells and has been shown to increase IL-6 secretion[187]. Recent work has shown that stromal cells derived from myeloma patients are able to support growth and proliferation of side population cells (possible stem cells) and this interaction is partially abrogated by down regulation of CXCR4 using plerixafor[95,195].

6.2 Aims

Plasma cells adhere to cells in the microenvironment, matrix proteins which leads to activation of signalling pathways and enhanced proliferation and resistance to therapy. The aim of these experiments is to study the adhesion of plasma cells to matrix proteins, particularly fibronectin which is a ligand for alpha 4 beta 1 integrin receptors expressed on plasma cells. Activation of the integrins using divalent cations mimics inside- out integrin activation resulting in the active conformation of integrins and leading to enhanced adhesion. In these experiments drugs were added to determine whether they can overcome integrin activation initiated by the cations. Plasma cells were also left to adhere on human bone marrow stromal cells and osteoclasts derived from primary cell through long term cultures. The effect of drugs on adhesion of plasma cells on the cellular substrata was analysed. Integrin activation upon adhesion in the plasma cells leads to activation of downstream signalling pathways. Western blot analysis of cells left to adhere with or without drugs were performed to analyse the pathways involved in adhesion and also elucidate the reasons for reduction in adhesion upon exposure to drugs.

6.3 Adhesion on extracellular matrix protein fibronectin

Fibronectin an extracellular matrix protein is secreted in soluble dimer form by myofibroblasts in the bone marrow. Upon activation by engagement with integrin receptors on the cell surface they are converted to insoluble multimeric fibrils. Fibronectin apart from binding integrins also binds collagen, glycosaminoglycans, fibrin and fibronectin itself[196]. Plasma cells were plated on fibronectin where the adhesion of plasma cells is dependent on integrins and also on polylysine where the adhesion is non integrin mediated. This was performed to study if the possible enhanced adhesion to the coated wells in the plate in response to integrin activation was non specific or integrin dependent. Integrins were fully activated on plasma cells by addition of magnesium EGTA to the cell suspension.

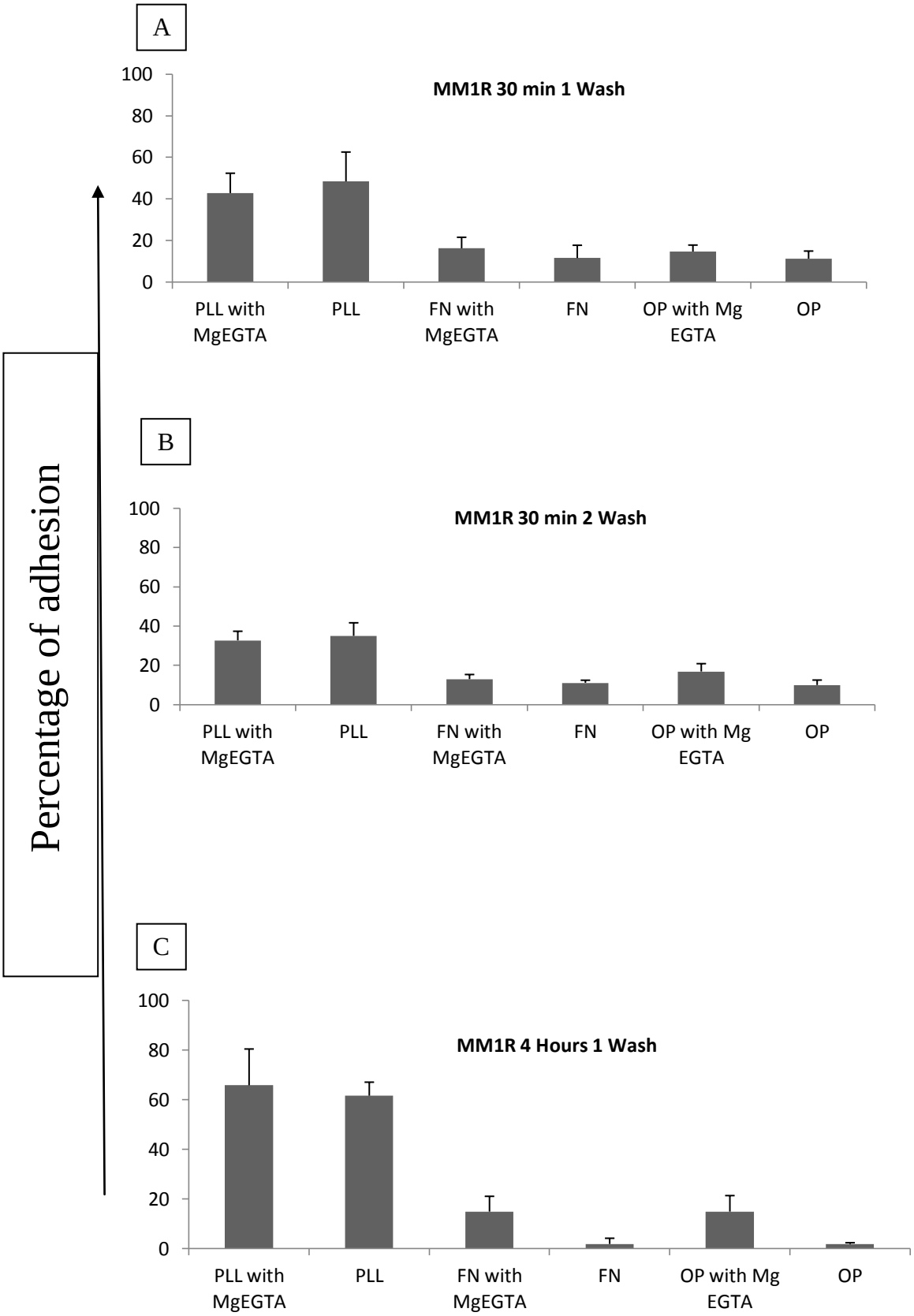
Adhesion assay calibration:

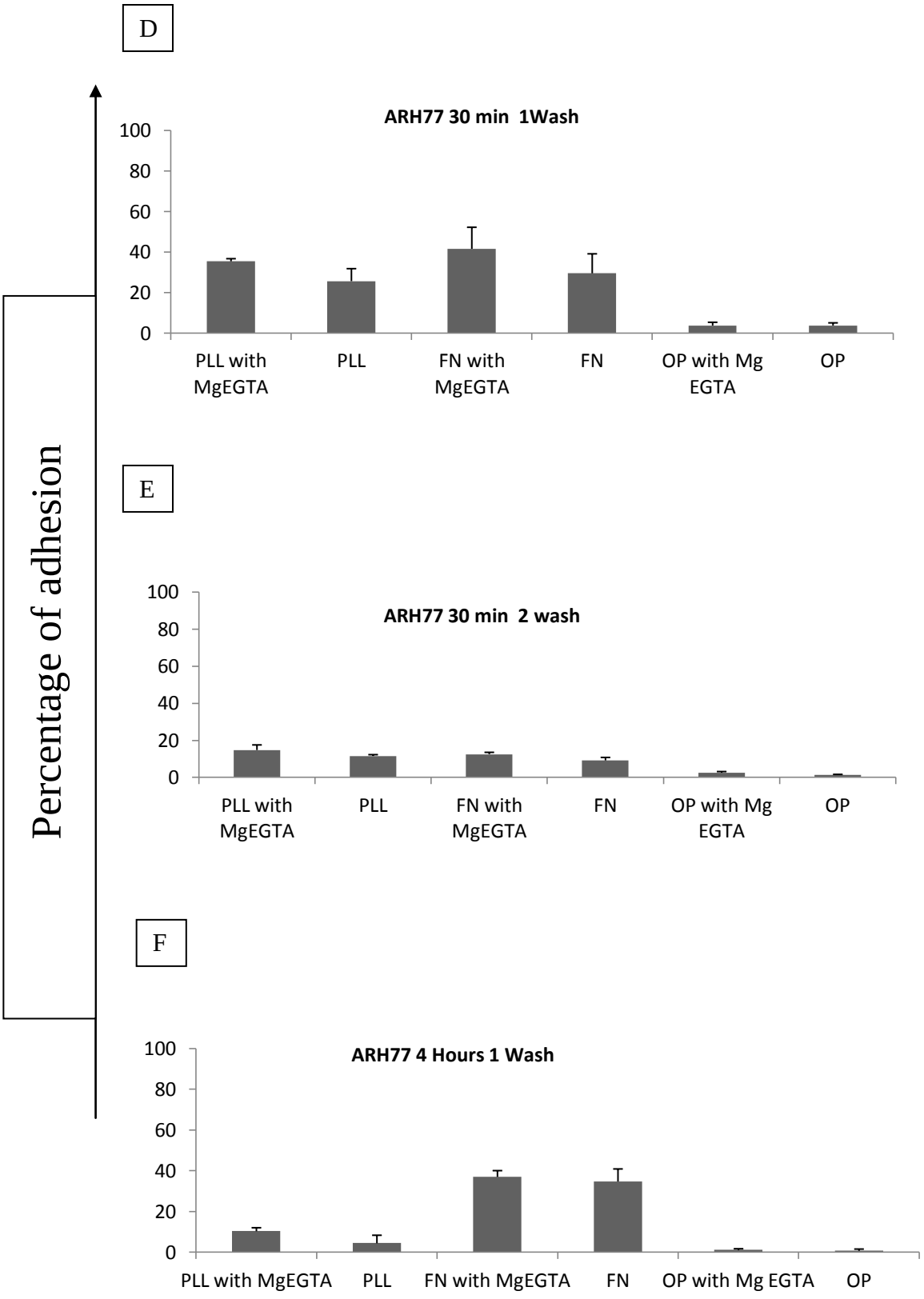
To standardise the adhesion assay, 2 human myeloma cell lines ARH77 and MM1R were taken. The wells in a 96-well plate were coated with polylysine, fibronectin and osteopontin. Myeloma cell lines were plated on precoated plates in media alone without serum. Equal volumes of cells were added in the experiment and control wells. Cells were left to adhere for either 30 minutes or 4 hours at 37°C in an incubator. After the period of adhesion, cells in suspension were removed and the plates were washed with phosphate buffered saline either once or twice whereas the control wells (whose values will correspond to the initial numbers of seeded cells) were left undisturbed. MTT analysis was performed as described before in methods section. The results showed that the plasma cell adhered to the substrata by the 30 minute

time period and level of adhesion was largely sustained only minor changes by the 4 hour time point (Figure 1). The 30 minute time point was the best time point to determine the level of adhesion. This is particularly important when drugs were added to the media during adhesion to pick up significant differences in levels of adhesion.

The number of washes required to remove the loosely adherent cells was also analysed. In MM1R cell lines no significant difference was observed between 2 washes and 1 wash with PBS. Although in ARH77 cell line the loosely adherent cells were further removed by an extra wash with PBS was significant the proportionate differences between the integrin activated wells and different substrata were retained (Fig 1). Due to the observed weak adherent nature of MM cells in short time periods (hours) one wash was chosen for further experiments. To standardise the readings obtained across all cell lines with and without drugs, 30 min adhesion time period with 1 wash with PBS after aspirating the media was used in all further experiments.

Fig 1: Adhesion calibration of Human Myeloma cell lines. Cells were plated on wells pre coated with 10ug/ml poly-L-lysine (PLL), Fibronectin (FN) and 10ug/ml osteopontin (OP) overnight. Plasma cells were left in serum free media to adhere for 30min or 4 hours. Loosely adherent cells were aspirated and washed once or twice with PBS and the adhered cells measured using a MTT assay. MM1R (A, B, C) ARH77 (C,D, E)

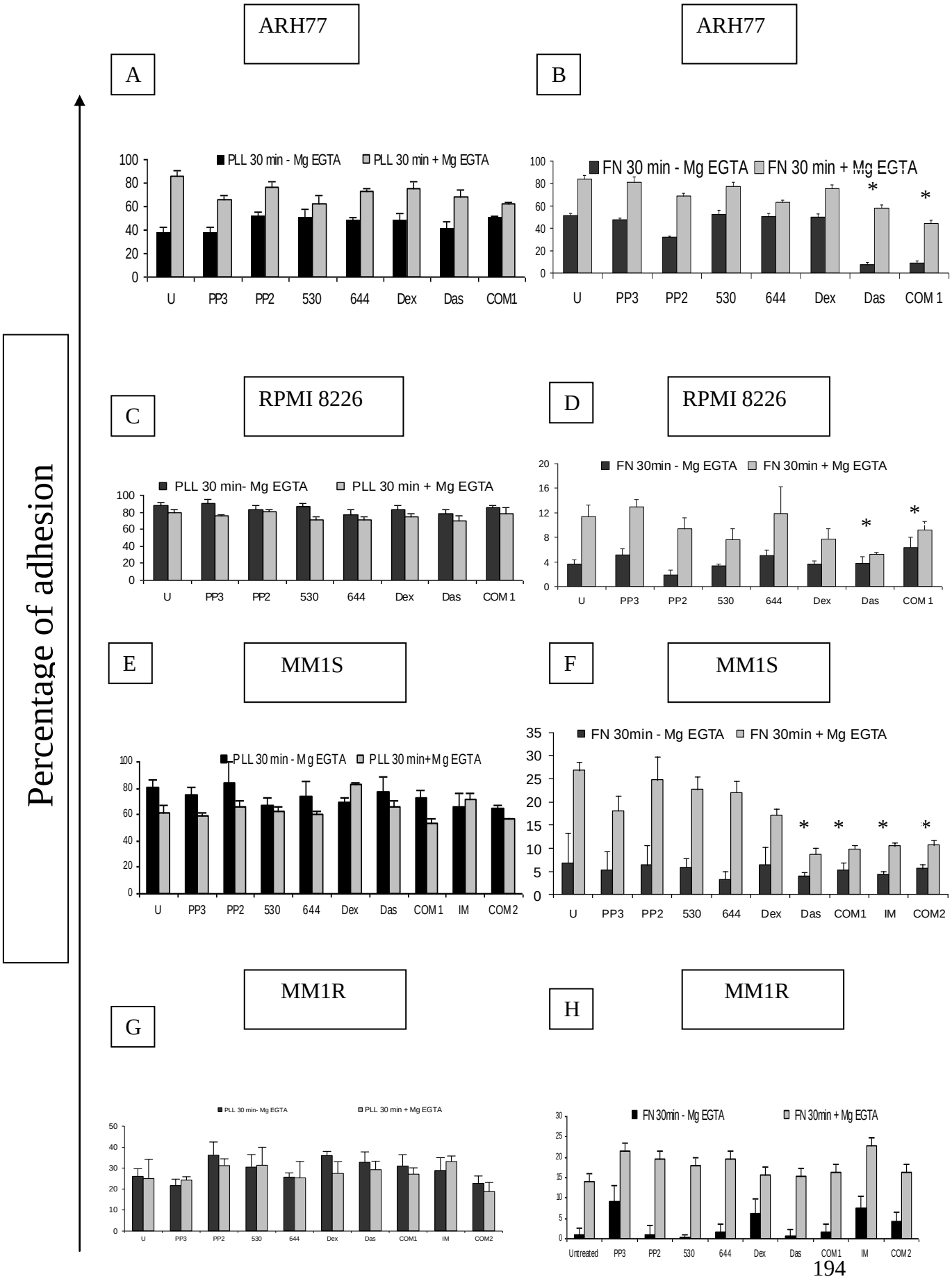

A
MM1R 30 min 1 Wash
100
80
60
40
20
0
PLL with MgEGTA
PLL
FN with MgEGTA
FN
OP with Mg EGTA
OP
B
MM1R 30 min 2 Wash
100
80
60
40
20
0
PLL with MgEGTA
PLL
FN with MgEGTA
FN
OP with Mg EGTA
OP
C
MM1R 4 Hours 1 Wash
100
80
60
40
20
0
PLL with MgEGTA
PLL
FN with MgEGTA
FN
OP with Mg EGTA
OP
Percentage of adhesion


D
ARH77 30 min 1Wash
100
80
60
40
20
0
PLL with MgEGTA
PLL
FN with MgEGTA
FN
OP with Mg EGTA
OP
E
ARH77 30 min 2 wash
100
80
60
40
20
0
PLL with MgEGTA
PLL
FN with MgEGTA
FN
OP with Mg EGTA
OP
F
ARH77 4 Hours 1 Wash
100
80
60
40
20
0
PLL with MgEGTA
PLL
FN with MgEGTA
FN
OP with Mg EGTA
OP
Percentage of adhesion

Human myeloma cell lines MM1S, MM1R, RPMI 8226 adhered more on polylysine than on fibronectin. The level of cell adhesion on polylysine ranged between 30-80% of cells plated (Figure 2). Binding on polylysine is due to the electrostatic interaction between the negative charge of the cell membrane and the positive charge of Lys, which makes it a very suitable substrate to induce high levels of cell adhesion on supports for culture without specific mediation of cell adhesion molecules. As expected, apart from ARH77 and murine myeloma cell lines, there was no significant enhancement of HMCL adhesion with addition of magnesium EGTA on polylysine. Although the reasons are not clear ARH77 is an adherent cell line with EBV infection which could explain the difference in adhesion observed on polylysine. The basal level of adhesion of all human myeloma cell lines on fibronectin was between 5 and 20% except for ARH77 cells where adhesion was between 40% and 80%. The percentage of adhesion of all human myeloma cell lines on fibronectin increased with the addition of magnesium EGTA (Figure 2 B, D, F, H). This is expected as EGTA chelates the calcium in the cells and magnesium binds the integrin and activates its affinity and subsequently avidity. Dexamethasone had no significant or very modest impact on adhesion on the human myeloma cell lines tested. Dasatinib and combination of dasatinib and dexamethasone inhibited the adhesion of the plasma cells on FN in all human myeloma cell lines except for MM1.R. Activation of integrins with divalent cations enhanced adhesion of these cells on FN but the levels of adhesion remained significantly lower in the dasatinib and dasatinib and dexamethasone combination treatment wells. Blockade of adhesion by Dasatinib was sustained even in the presence of dexamethasone. Activation

of integrins in MM1R, a dexamethasone resistant cell line was able to overcome the adhesion inhibition effects of drug combination (Fig 2). Adhesion inhibition observed in dasatinib was significantly higher than observed in PP2 and AZD0530, which are Src inhibitors. Imatinib also inhibited adhesion of MM1S cells on FN. This observation indicates that the effects of dasatinib on adhesion are mediated in part through multiple intracellular signalling pathways involving Src and c-Abl kinases.

Fig 2: Effect of Drug treatments on Adhesion of Human Myeloma cell lines. Cells were plated on wells pre coated with poly-L-lysine and Fibronectin overnight, in serum free media with drugs for 30min. Loosely adherent cells were aspirated and the adhered cells measured using a MTT assay. ARH77 (A,B), RPMI8226 (C,D), MM1S (E,F) and MM1R (G,H). Histograms show Mean +SD. Drug Treatments - 10uM PP3, 10uM PP2, 0.1uM AZD0530 Src inhibitor (530), 0.5uM AZD0644 ERK inhibitor (644), COM and COM1 represent a combination of 150nM Dasatinib and 2uM Dexamethasone and COM2 is a combination of 10uM Imatinib and 2uM Dexamethasone. * p<.005 student t-test


ARH77
A
PLL 30 min - Mg EGTA
PLL 30 min + Mg EGTA
U
PP3
PP2
530
644
Dex
Das
COM1
ARH77
B
FN 30 min - Mg EGTA
FN 30 min + Mg EGTA
COM 1
RPMI 8226
C
PLL 30 min- Mg EGTA
PLL 30 min + Mg EGTA
RPMI 8226
D
FN 30min - Mg EGTA
FN 30min + Mg EGTA
MM1S
E
PLL 30 min - Mg EGTA
PLL 30 min+Mg EGTA
COM 1
IM
COM 2
MM1S
F
FN 30min - Mg EGTA
FN 30min + Mg EGTA
COM1
COM2
MM1R
G
MM1R
H
Untreated
Percentage of adhesion

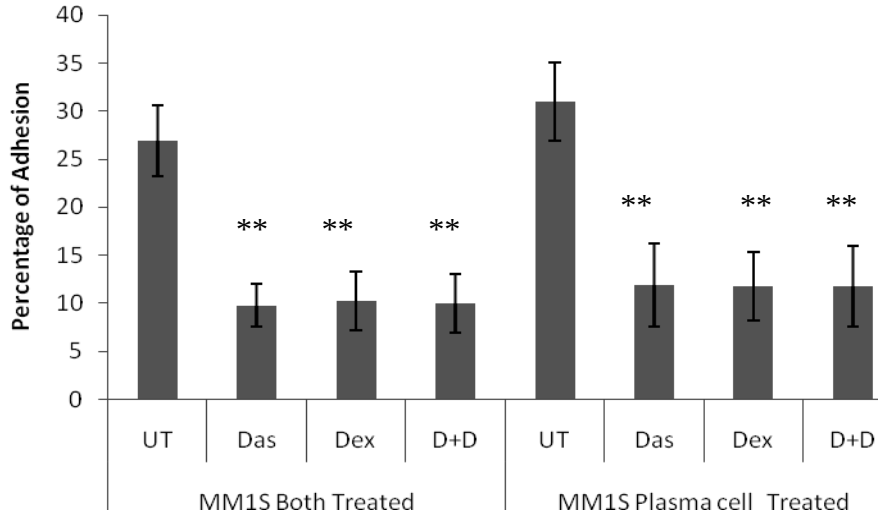
6.4 Adhesion of plasma cells on HS5 Stromal cells

Plasma cells adhere to stromal cells in vivo and this interaction will enhance the protection rendered by the stromal cells and leads to drug resistance. The interaction between stromal cells and plasma cells increases the secretion of IL-6 in a paracrine fashion. Adhesion assay protocol used for adhesion of myeloma cells on fibronectin is not applicable as the signal generated by MTT assay will be from both cell groups. As described in chapter 5, the eGFP positive myeloma cells were used in the adhesion assays on HS5 stromal cells with or without drugs to assess response.

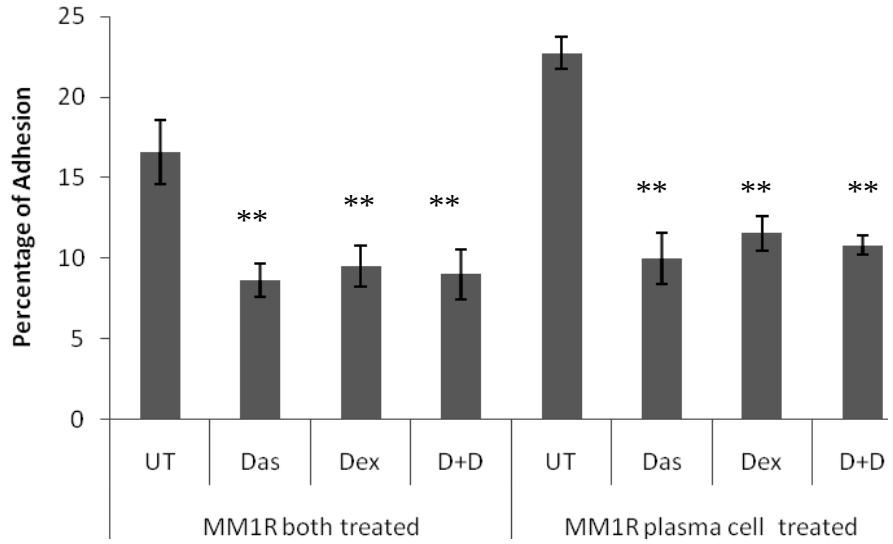
HS-5 cells were added to 96 well plate and left to spread and proliferate overnight adequate enough to cover more than 90% of the base of the wells. Plasma cells were layered on the stromal cells in serum free media with and without drugs for an hour. Supernatants were aspirated and the fluorescence intensity of the adherent plasma cells was measured relative to unaspirated wells using a fluorescent plate reader. The plasma cells alone were pre-treated before layering on to the stromal cells and compared with adding drugs to the coculture or plasma cells and stromal cells during adhesion. Treatment with Dasatinib (150nm), dexamethasone (1uM) and the drug combination significantly reduced the adhesion of plasma cells on HS5 stromal cells. There was up to 60% reduction in adhesion in both MM1S and MM1R cell lines (Fig 3). There were no significant differences between the results obtained when the plasma cells were treated alone before the adhesion assay or treatment of the cocultures during adhesion. This confirms that the reduction in adhesion observed is due to the effect of the drugs dexamethasone and dasatinib on the plasma cells.

Fig 3: Treatment with Dexamethasone and Dasatinib directly impairs the adhesion of MM cells on stromal cells. Histograms showing the percentage of adhesion of MM cells on stromal cells in the presence or drugs. Cells were left untreated (U) or treated for the period of adhesion of an hour with 1 uM Dexamethasone (Dex), 150 nM Dasatinib (Das) or the combination of these 2 drugs (DD). In the plasma cells only treated group the cells were pre-treated with the drugs before the period of adhesion. (A) MM1S and (B) MM1R. The assay was repeated at least 4 times and results were reproducible. Histograms show mean percentage of adhesion +/- SD. ** p<.005 student t-test.

A


Percentage of Adhesion
40
35
30
25
20
15
10
5
0
**
**
**
**
**
**
UT
Das
Dex
D+D
UT
Das
Dex
D+D
MM1S Both Treated
MM1S Plasma cell Treated

B


Percentage of Adhesion
25
20
15
10
5
0
**
**
**
**
**
**
UT
Das
Dex
D+D
UT
Das
Dex
D+D
MM1R both treated
MM1R plasma cell treated

6.5 Adhesion of plasma cells on osteoclasts

The presence of osteoclastic activation in bone marrows of myeloma patients is fairly universal**.** Osteoclast mediated plasma cell survival has been shown to be mediated through direct cell - cell contact. As described in the methods section primary bone marrow cultures from myeloma patients were treated with colony stimulating factor and RANK ligand for 3 weeks to form osteoclasts. Plasma cells were added on to fully formed osteoclasts and left to adhere for 60 minutes in serum free media. The plasma cells were either pre-treated with drugs or the drugs were added to the coculture during the period of adhesion. Short term adhesion experiments were chosen as osteoclast being cells of macrophage lineage will phagocytose dying plasma cells and also form fusion nuclei with plasma cells as has been described before. As in the case of adhesion assay on stromal cells a fluorescent marker is required to measure the level of adhesion as MTT assays will calculate the signal produced from the whole coculture.

The initial assays were performed by CFSE labelling of plasma cells as described in the methods section of the thesis. When the plasma cells labelled with CFSE were analysed by flow cytometry a uniform peak in the FITC channel was observed (Fig 4). Cells were washed twice to remove any additional dye. CFSE labelled plasma cells were added to the osteoclast cultures and fluorescence intensity was measured after the loosely adherent cells were removed by aspiration and wash with PBS. The experiments were reproducible and fluorescence intensity emitted by the CFSE labelled plasma cells were markedly higher than the background fluorescence emitted by the osteoclasts in the base of the wells.

Fig 4: CFSE labelling of plasma cells for adhesion experiments. Myeloma cell line MM1S was labelled with CFSE (Vybrant CFDA SE Tracer kit, Invitrogen, UK) as per protocol. Flow cytometry was performed to confirm CFSE labelling of the myeloma cells.

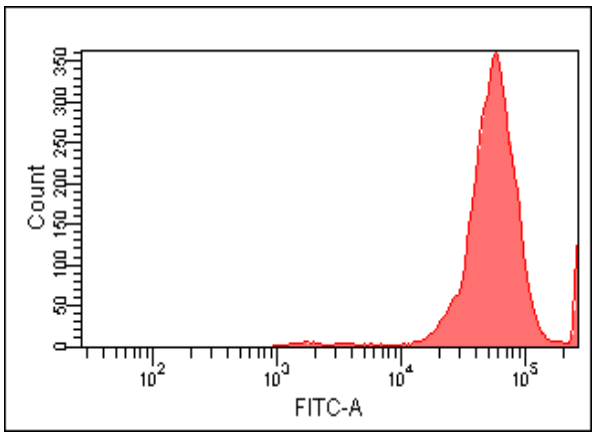
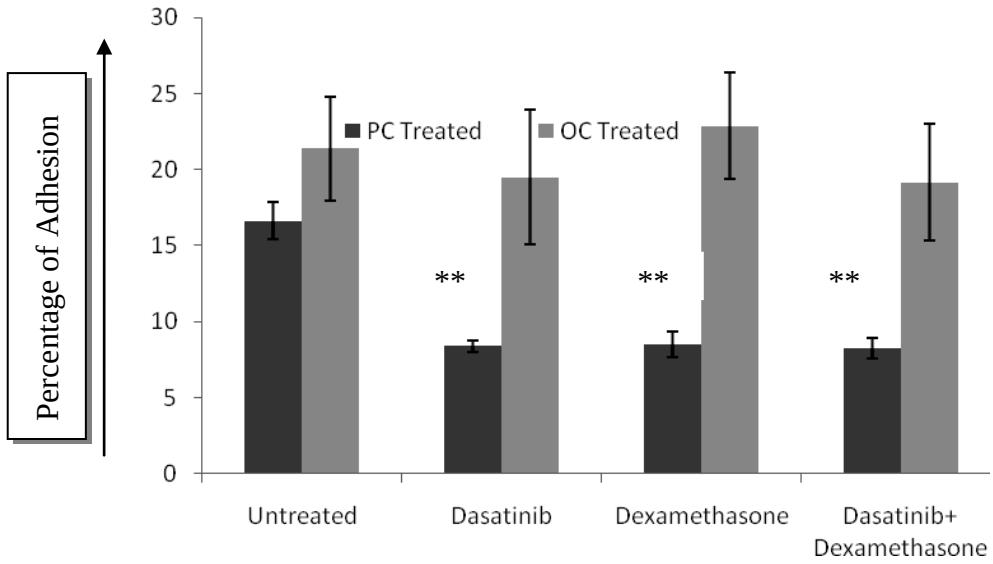


Fig 5: Dasatinib and Dexamethasone reduce adhesion of plasma cells on Osteoclasts. CFSE labelled MM1.S plasma cells alone or osteoclasts alone were preincubated with drugs for 60 minutes prior to plating on osteoclasts and adhesion was measured using fluorescent plate reader. Histograms show Mean percentage adhesion +SD. ** $p < 0.005$ student t-test

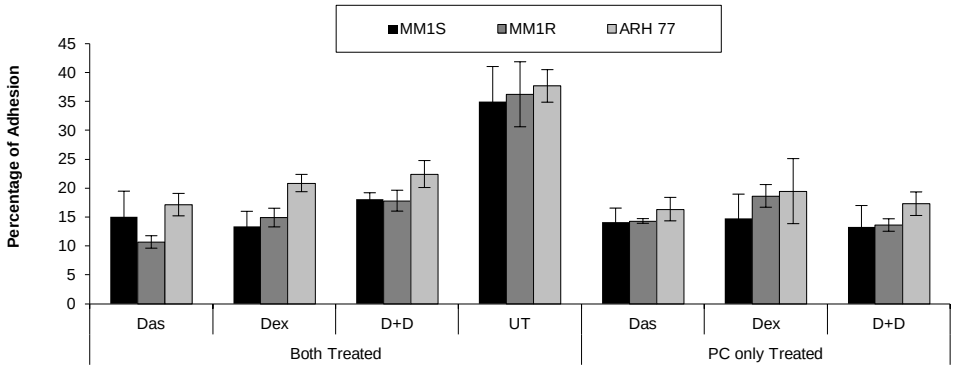


To understand if the reduction in adhesion observed with the drug treatments is due an effect on the osteoclasts or the plasma cells, pre-treatment with drugs of both osteoclasts and plasma cells were carried out in parallel. Adhesion of MM1S plasma cells pre-treated with dexamethasone, dasatinib or the combination was significantly lower compared to plasma cells not exposed to drugs. Treatment of osteoclasts with did not affect the adhesion of plasma cells in the short term adhesion assay. Treatment with both dasatinib and dexamethasone had an equivalent reduction in adhesion of plasma cells by about 50% in comparison with cells left untreated. The short term combination pre-treatment with dasatinib and dexamethasone did not further decrease adhesion of plasma cells on osteoclasts than either of the drugs alone (Fig 5).

As described in chapter 5 of this thesis eGFP transduced plasma cell clone of MM1S, MM1R and ARH77 cells were generated. Osteoclasts derived from primary samples were grown in 96 well plates. Previously it was observed with CFSE labelled plasma cells, that there was very little effect on adhesion of plasma cells when osteoclasts were treated alone (Fig 6). In this experiment either the plasma cells only were pre-treated before adhesion, or both the osteoclasts, plasma cells coculture were treated during the period of adhesion. The adhesion efficiency of MM1S, MM1R and ARH77 human myeloma cells significantly decreased upon treatment with dasatinib and dexamethasone. Treatment of both plasma cells and the osteoclasts during the period of adhesion did not further reduce the level of adhesion. This shows that the drug treatment decreased the adhesion efficiency of plasma cells but the mechanisms mediating this are not clear from this experiment.

The combined treatment of dasatinib and dexamethasone in a short period did not further reduce the percentage of adhesion than either of the drugs alone. This was also observed when plasma cells were left to adhere on stromal cells. ARH77 cell line was chosen for adhesion experiments as it showed high efficiency of adhesion on fibronectin in comparison to other myeloma cell lines tested. It was striking to see that the adhesion efficiency of these cells were no higher than MM1S and MM1R cell lines. The level of short adhesion across stromal cells, ECM protein fibronectin and osteoclasts was inefficient, particularly fibronectin. What is not clear is whether in the in vivo setting where humoral factors also play a role, and there is abundance of these cells and matrix proteins it is possible that plasma cells and its multiple receptors systems could function differently.

Figure 6. C**ell adhesion efficiency of MM cells on osteoclasts in response to treatment with Dexamethasone and Dasatinib**. eGFP expressing plasma cells were left untreated (UT) or were treated with 1 uM Dexamethasone (Dex), 150 nM Dasatinib (Das) or the combination of these 2 drugs for 1 h during the course of the adhesion assay (PC + OC treated) or alternatively, pre-treated with drugs for 3 hours prior the adhesion assay (PC only treated). Using a fluorescent plate reader, the number adhered cells was estimated by measuring the average of fluorescent intensity per well after adhesion. Histograms show the percentage of adhered MM cells after one h incubation on osteoclasts differentiated for 17 days.



6.6 Adhesion signalling pathways

Integrins are the major class of receptor systems involved in the adhesion of plasma cells on the ECM. The receptors involved in the adhesion of plasma cells on stromal cells and osteoclasts are less clear, although integrins do play a role. The adhesion on ECM protein is much better defined particularly the interaction with ligands which use integrins to adhere, stimulate and coordinate other intracellular processes. For the purpose of my thesis I was interested in studying the downstream signalling pathways involved during integrin activation upon adhesion to ECM protein fibronectin. Treatment with drugs and resultant changes in the signalling pathways with drug exposure has been studied.

Integrin activation by ligand binding, results in the oligomerisation of FAK (Focal adhesion kinase), which is mediated by Talin. Autophosphorylation of FAK at residue Tyr397 results in the binding of SH2 domain of Src and Fyn which phosphorylates a number of FAK-associated proteins including paxillin. Phosphorylation of Tyr397 also leads to the recruitment of other SH2-containing proteins; including the PI3K (Phosphatidyl Inositol-3Kinase). Src can also phosphorylate FAK at tyrosine residue 925, creating a binding site for the growth-factor-receptor-bound protein complex, the GRB2-SOS complex and activate another small GTPase, Ras. Activated Src also phosphorylates CAS (Crk-Associated Substrate), enabling it to bind Crk leading to an increase in the affinity of the membranes for Rac [197]. This underlines the importance of Src as a downstream signalling protein in integrin activation. c-Abl kinase is also activated downstream upon engagement of ligand such as fibronectin

with the integrins resulting in activation of MAPK. C-Abl also phosphorylates the cytoskeletal protein paxillin, a component of focal adhesion. In neutrophils, c-Abl kinase is activated after β_2 integrin engagement. β_2 integrin engagement induces recruitment of c-Abl kinase to β_2 integrin cytoplasmic domain via talin head, a cytoskeletal protein. c-Abl kinase also regulates signalling protein Vav activity [198]. In these experiments the activation of c-Abl and Src kinase activity downstream of integrin receptor activation has been studied in the plasma cells adhering to fibronectin.

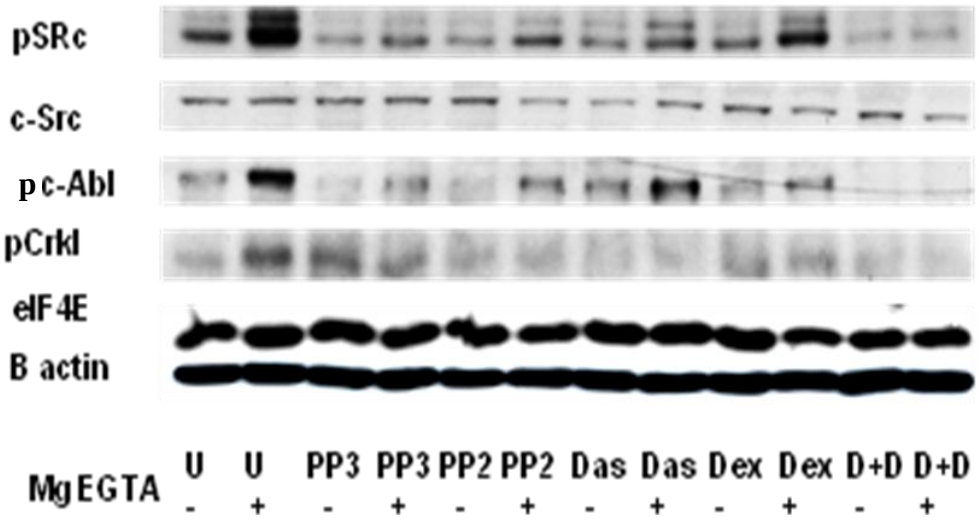
Plasma cells upon short term adhesion to fibronectin were lysed and western blots were performed to analyse the signalling pathways in these cells upon integrin activation with magnesium and EGTA. Plasma cells were also treated with the drugs dasatinib and dexamethasone alone or in combination during the period of adhesion. The expression and activation of proteins downstream of integrin activation was compared with cells left untreated.

Src and c-Abl phosphorylation was observed when the plasma cells adhered to polylysine in the wells. Crk like protein (Crkl) phosphorylation which is a surrogate marker for Abl tyrosine kinase activity was also up regulated with adhesion (Fig 7). This shows that even on surfaces lacking integrin, there is an activation of Src and Abl. Treatment with src inhibitor PP2, downregulated the phosphorylation of Src in contrast with cells left untreated and treated with Mg EGTA. Dasatinib also inhibited the phosphorylation of Src comparable to PP2. Although when integrins were fully activated MgEGTA in MM cells both Dasatinib and PP2 inhibited Src and c-Abl signalling, the net Src/c-Abl active signal was higher compared with drug-treated but not MgEGTA activated

cells. Dexamethasone induced a minor decrease in the phosphorylation of c-Src but the combination of dasatinib and dexamethasone were able to very effectively down-regulate integrin activation as evidenced by the lower levels of phosphorylation of Src and c-Abl on the immunoblot despite activation of integrins by Mg EGTA (Fig 7 A). C-Abl phosphorylation increased when cells were treated with MgEGTA. But dexamethasone and dasatinib both downregulated C-Abl activity and the combination almost completely inhibited phosphorylation of the Abl protein (Fig 7). This was supported by a reduction in levels of phosphorylated Crkl Protein. These results were quantified by the measurement of the intensity of the bands over the respective loading controls (Fig 7 B).

Fig 7: Effect of Dasatinib on integrin signalling in ARH 77 Myeloma cell line. Lysates obtained from ARH77 cells plated on PLL (A) +/- integrin activation with Mg EGTA, after drug treatment for 30 minutes. 10uM PP3, 10uM PP2, 10uM 150nM Dasatinib (Das), 2uM Dexamethasone, 150nM +2uM Dasatinib + Dexamethasone (D+D). Membranes were immunoblotted for pSrc, Phospho c-Abl, Src and ß actin as loading control. C- Abl activity immunoblot kit containing Phospho c-Abl, pCrkl and e14AE as positive control was used. Figures B represents Intensity of Band / Loading control ratio in arbitrary units of Figures A.

A


pSRc
c-Src
p c-Abl
pCrkl
eIF4E
B actin
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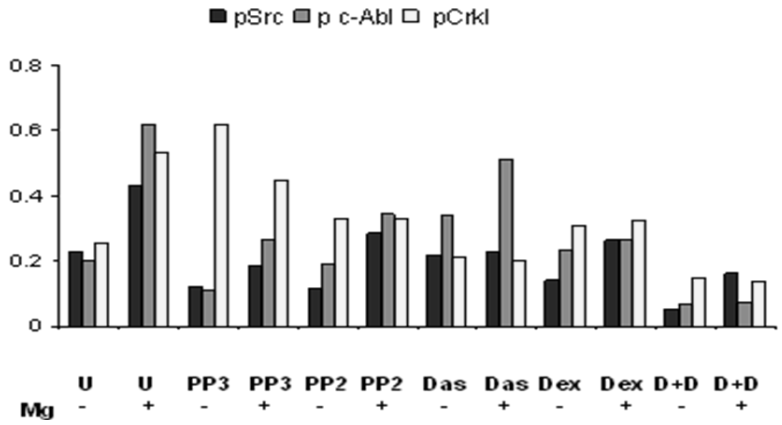
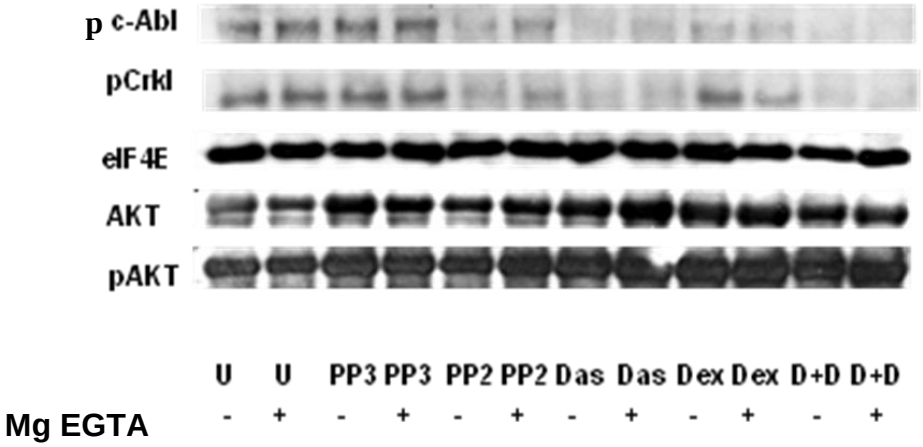
B


pSrc
p c-Abl
pCrkl
0.8
0.6
0.4
0.2
0
U U PP3 PP3 PP2 PP2 Das Das Dex Dex D+D D+D
Mg - + - + - + - + - + - +

On Fibronectin, where plasma cell adhesion is integrin dependent in the untreated cells there was clear evidence of Src, Abl phosphorylation, AKT phosphorylation and pCrkl observed on the immunoblots (Fig 8). Upon treatment with PP2 there was reduction in Src, Abl and Crkl phosphorylation which was not observed with PP3, a PP2 analogue without the inhibitory activity. Treatment with dexamethasone reduced the levels of phosphorylated Abl and Crkl without any effect on the levels of phosphorylated AKT. With Dasatinib treatment the levels of phosphorylation of Src, c-Abl and Crkl were almost undetectable with no effect on the AKT pathway (Fig 8). The combination of Dasatinib and dexamethasone completely inhibited the Abl and Src kinase pathways. In the figure 8 C the ratio of the phosphorylated proteins to the loading control shows objectively the significant reduction in Src and Abl kinase activation with Dasatinib on its own and with the combination of dexamethasone.

Figure 8: Effect of Dasatinib on integrin signalling in ARH 77 Myeloma cell line. Lysates obtained from ARH77 cells plated on FN (A),(B) +/- integrin activation with Mg EGTA, after drug treatment for 30 minutes. 10uM PP3, 10uM PP2, 10uM 150nM Dasatinib (Das), 2uM Dexamethasone, 150nM +2uM Dasatinib + Dexamethasone (D+D). Membranes were immunoblotted for pSrc, Phospho c-Abl, Src and ß actin as loading control. C- Abl activity immunoblot kit containing Phospho c-Abl, p Crkl and e14AE as positive control was used (Fig A). Figures C represents Intensity of Band / Loading control ratio in arbitrary units of Figures A.

A


p c-Abl
pCrkl
eIF4E
AKT
pAKT
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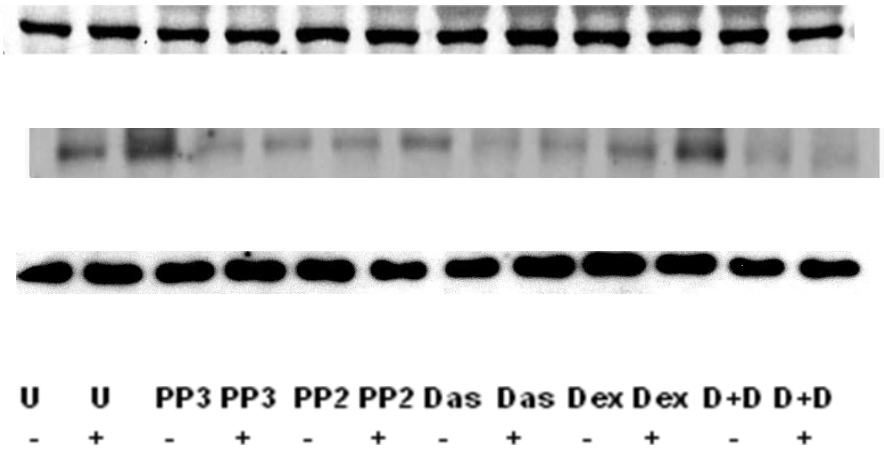
Mg EGTA
- + - + - + - + - + - +

B


C-Src
p Src
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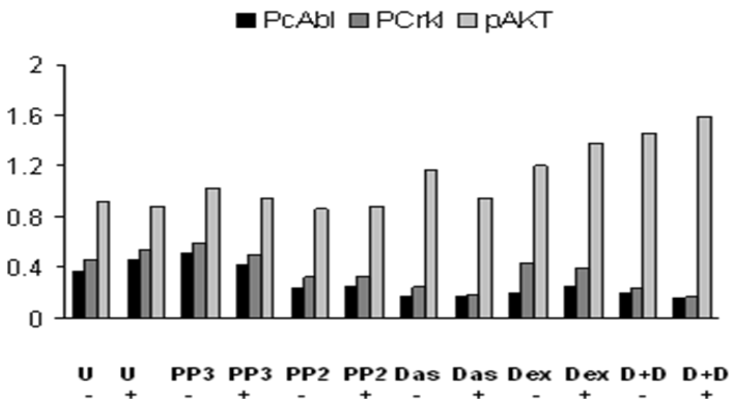
U U PP3 PP3 PP2 PP2 Das Das Dex Dex D+D D+D
- + - + - + - + - + - +

C


PcAbl PCrkl pAKT
2
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- + - + - + - + - + - +

6.7 Discussion

Plasma cell adhesion to the extracellular matrix proteins and cells in the bone marrow microenvironment plays an important role in survival of plasma cells and slow relapse following therapy. Plasma cell adhesion on stromal cells and bidirectional interaction leads to disease growth and progression. Although the changes in the plasma cell compartment growing in the presence of the stromal cells have been well studied, the changes in the stromal cell compartment still remains poorly understood. Although myeloma cell lines have been shown to proliferate with stromal cell cultured medium, Plasma cell lines exclusively dependent on cell-cell contact with bone marrow stromal cells have been described[199,200]. I observed that level of adhesion on fibronectin; an extracellular matrix protein present in abundance in the marrow was variable between the different cell lines. This could be explained by the variability in expression of integrin receptors as has been described in primary cells when the disease evolves[75]. Integrins can also be activated in the presence of soluble mediators and there is preferential use of sets of dimeric integrin receptors depending on the extracellular matrix protein in context [201-203]. Also, local concentration of cytokines could be different in the bone marrow. These factors have to be taken into consideration when interpreting in vitro results and how they correlate with what maybe happening in vivo. Dasatinib down regulated the adhesion of plasma cells on fibronectin. The effect was more obvious on myeloma cells at basal level of integrin activation. Inhibition of adhesion by Dasatinib was not observed in all the cell lines. Various factors can explain the heterogeneity of response to dasatinib between the myeloma cell lines. Genomic changes in the plasma cells may

lead to heterogeneity in adhesion to fibronectin as described before[204]. Additionally, adhesion on extracellular matrix protein such as fibronectin is also mediated by syndecan-1, CD44 variants and RHAMM which mediate interactions with hyaluronan, and the SDF-1/CXCR4 axis. The expression and activity of these molecules are regulated by cytokines within the bone marrow microenvironment. It is not clear at present as to which of these sets of adhesion processes is key for survival of plasma cells, although adhesion of plasma cells on fibronectin does lead to drug resistance as shown by Dalton et al. More recently, this group have shown that Notch activation during adhesion of myeloma cells on stromal cells leads to arrest of myeloma cells in G0/G1 phase of cell cycle with upregulation of p21[205]. In my experiments dasatinib at nanomolar concentrations inhibited both c-Src and c-Abl signalling pathways downstream of integrins when plasma cells were adhered on to fibronectin. Src is a key tyrosine kinase and adaptor protein downstream in the integrin signalling pathway. It functions as a non receptor tyrosine kinase which is can bind Grb2 and activate MAPK kinase during integrin activation, a process that can be inhibited by dasatinib. In my experiments, downregulation of both c-Src and c-Abl mediates the reduction in adhesion of MM cells. It is well known that Src can activate c-Abl and they also interact downstream of integrin signalling pathways in cancer cells [206]. The downregulation of c-Abl activity seen in our experiments with PP2 a Src kinase inhibitor could be explained by this interaction. The inhibition of integrin activity by Dasatinib is further supported by a recent study looking at non invasive photon emission tomography (PET) monitoring of $\alpha_v\beta_3$ activity in tumours using cyclic pentapeptides containing the RGD peptide sequence.

When mice containing $\alpha_v\beta_3$ positive tumours were treated with dasatinib, PET activity significantly decreased within 72 hours suggesting integrin inactivation and prolonged therapy led to tumour reduction[207].

Multiple mechanisms have been described for Dexamethasone resistance myeloma patients. One of the mechanisms is adhesion of myeloma cells to stromal cells though expression of VLA-4 or CD44 engagement [89]. Bortezomib had been shown to overcome the resistance mediated through CD49d[208] . My findings show that Dasatinib can overcome Dexamethasone CAM-DR mediated by the interaction of MM cells with the cellular and non-cellular components of the BM microenvironment. My data suggest that Dasatinib would form an useful adjunct in treating myeloma patients exhibiting secondary Dexamethasone resistance.

EFFECT OF DASATINIB ON OSTEOCLASTS AND INTERACTION OF PLASMA CELLS WITH THE MICROENVIRONMENT

7. Introduction

Bone disease is a major cause of morbidity in myeloma. Clinical manifestations of osteoclast activation occur in up to 80% of patients with myeloma. Although treatment with antimyeloma agents induces stringent remission based on currently available clinical criteria, the effects of bone morbidity significantly impairs patient's quality of life. Osteoclasts are highly dependent on the intact cytoskeleton to be able to perform the function of bone resorption. Also osteoclasts within the bone marrow are under the effects of soluble factors and cellular components T cells and plasma cells. The interaction between plasma cells and osteoclasts has been described extensively in the literature. There are recent reports indicating that the immune system of the patient may cooperate with osteoclasts to enhance bone resorption in myeloma patients [209,210]. Soluble factors such DKK1 produced both by stromal cells and plasma cells inhibit osteoblastogenesis and activate osteoclasts. Recently, soluble decoy receptor 3 (DcR3) of the Tumour necrosis factor super receptor family has been described to activate osteoclasts. DcR3 transgenic mice have low bone mass and high osteoclast numbers with activated phenotype. Myeloma patients have high levels of DCR3 and both plasma cells and T lymphocytes produce DCR3. This results in an increase in the numbers of osteoclasts in the blood and marrow of patients[211]. It is critical to disrupt the osteoclasts cytoskeleton and interaction between plasma cells and osteoclasts to enhance the therapeutic effects of treating this condition. In this chapter, the effects of Dasatinib, dexamethasone on the osteoclast cytoskeleton, function and interaction with cells of the microenvironment have been studied.

During the course of disease stroma induced resistance plays a key role in determining response to therapy. Bone marrow stromal cell proliferation in response to addition of Dexamethasone has been demonstrate in vitro [212]. We wanted to analyse the effects of Dasatinib of myeloma cells cocultured with bone marrow stroma in the presence of Dexamethasone. We have developed a model to look at the differential effects of drugs on both the stromal cells and the myeloma cells in coculture with eGFP positive myeloma cells and mCherry- HS5 cells.

During the course of the experimental work for my thesis, another member of the laboratory developed mCherry-labelled-HS5 BM fibroblastic stromal cells. As in the case of eGFP-MM cells, the number of mCherry-HS5 cells can be evaluated by measuring the fluorescence intensity emitted by the cells (λex = 587; λem = 610). By employing mCherry-labelled-HS5 BM fibroblastic stromal cells co-cultured in direct contact with eGFP-labelled MM cell lines we were enabled to accurate study the heterotypic cell interactions in both cell compartments. This fluorescent-based platform can assess the impact of drugs on stromal and tumour cell proliferation and viability. This experimental model provides a significant improvement on previous methodologies by allowing for the concomitant assessment of tumour and BM stromal responses to anticancer drugs.

7.2 Aims

Osteoclasts derived from the primary bone marrow cells of patients as described in the methods section were analysed by microscopy and their function was assessed on rabbit dentine slices. Src is a key protein involved in osteoclast motility and function. Dasatinib was used in parallel with dexamethasone to study a) the effects on the cytoskeleton and function of osteoclasts. b) the effect of drugs on fibroblastic stromal cells c) the effect of drugs on plasma cells and BM microenvironmental cells in coculture. I wanted to validate the findings using the novel coculture system. The effects of the drugs on the viability of plasma cells when they were in the presence of protective osteoclasts and stromal cells were also studied.

7.3 Effects on osteoclastogenesis and the cytoskeleton

Primary bone marrow mononuclear cells from myeloma patients were differentiated into osteoclasts. By day 17, the mature osteoclasts are fully differentiated with capability to resorb bone. Osteoclasts were exposed to drugs from day 12 or left untreated. Using Immunofluoroscence, the morphological features of osteoclast cytoskeletal structure were analysed. In the cells left untreated large multinucleated osteoclasts were formed with actin ring in the periphery of the cell (Fig 1 Panel A). On higher magnification, the podosome assembly forming actin rings in the periphery of the osteoclast was seen (Fig 1 Panel E). Treatment with dexamethasone had no significant effect on the size of the osteoclast, actin ring formation or podosome assembly (Fig 1 Panel C). Dasatinib at 150nM concentration resulted in formation of small clusters of actin at the periphery of the cell, with the absence of uniform ring formation (Fig 1 Panel B, F). Treatment of the osteoclasts with the combination of dasatinib and dexamethasone showed impaired actin ring formation and clustering of actin in the cells (Fig 1 Panel D). In order to quantify the observed changes in size and nuclearity the osteoclasts grown on the cover slip were counted in a 10X field in triplicate and a mean number was plotted as a histogram (Fig 2 Panel A). The average size of osteoclast is about 340 microns in diameter and the size of the osteoclast increases after 11 days of plating[213].

On Day 12 when the cultures were untreated the number of osteoclasts per field equal between the experiment wells. The cultures left untreated by day 17, had lesser number of osteoclasts per field because of the increase in size of the cells (Fig 2 Panel A). The dexamethasone treated cells also continue to

increase in size in contrast with Dasatinib treatment which inhibited further increase in size of osteoclasts. Dasatinib also caused arrest of osteoclast fusion as evidenced by most cells having less than 4 nuclei (Fig 2 B). Cultures left untreated and treated with dexamethasone showed multinucleated osteoclasts (Fig 2 B). Treatment with the combination of dasatinib and dexamethasone inhibited osteoclast fusion and increase in size of the osteoclast. The effect on the osteoclasts was identical to those observed with Dasatinib alone (Fig 2 Panel A, B), which also prevented fusion of mononucleated immature osteoclasts forming multinucleate larger well differentiated and mature cells. This effect was reversible when cultures were fed with fresh media in the absence of drugs for 3 days (Fig 3)

To study the function of these osteoclasts differentiated from mononuclear cells obtained from myeloma patients, cultures were established on rabbit dentine slices. Osteoclasts resorb bone by forming sealing zones and ruffled border on the dentine surface. Once the osteoclasts form these membrane domains the proteolytic enzymes and collagenase are secreted on the bone surface to resorb the bone. We hypothesised that by the observed inhibition of osteoclast maturation and cytoskeletal integrity with Dasatinib and the combination of Dasatinib and dexamethasone there will be a significant reduction in bone resorption. The numbers of pits on the dentine slices were counted under 10X objective showing inhibition of bone resorption by Dasatinib (Fig 4 Panel E). In the untreated cultures, osteoclasts resorbed the dentine slices, as observed on phase contract microscopy (Fig 3 Panel A). In the dexamethasone treated cultures the osteoclasts resorbed the dentine slices evidenced by formation of resorption pits (Fig 4 Panel B). Treatment

with Dasatinib and the combination of Dasatinib and dexamethasone inhibited bone resorption evidenced by the lack of presence of resorption pits (Fig 4 Panel C, D).

Fig 1: Dasatinib reduces the size of the osteoclast and disrupts actin cytoskeletal architecture. Osteoclasts were seeded on glass cover slips and at the time of interest they were fixed with 1% PFA, permeabilised with 0.05% Triton X-100 and stained with Alexa568-Phalloidin to detect the distribution of actin filaments (red) and DAPI to detect nuclei (blue). Osteoclasts were left Untreated (A) or treated with 150nM Dasatinib (B), 1uM Dexamethasone (C), combination of 150nM Dasatinib and 1uM Dexamethasone (D) Structure of actin ring with clustered podosomes in an Untreated Osteoclast (E) , Disrupted actin aggregates in Osteoclasts treated with 150nM Dasatinib and 1uM Dexamethasone (F). Bar (A-D) 40µm, (E-F) 10 µm.

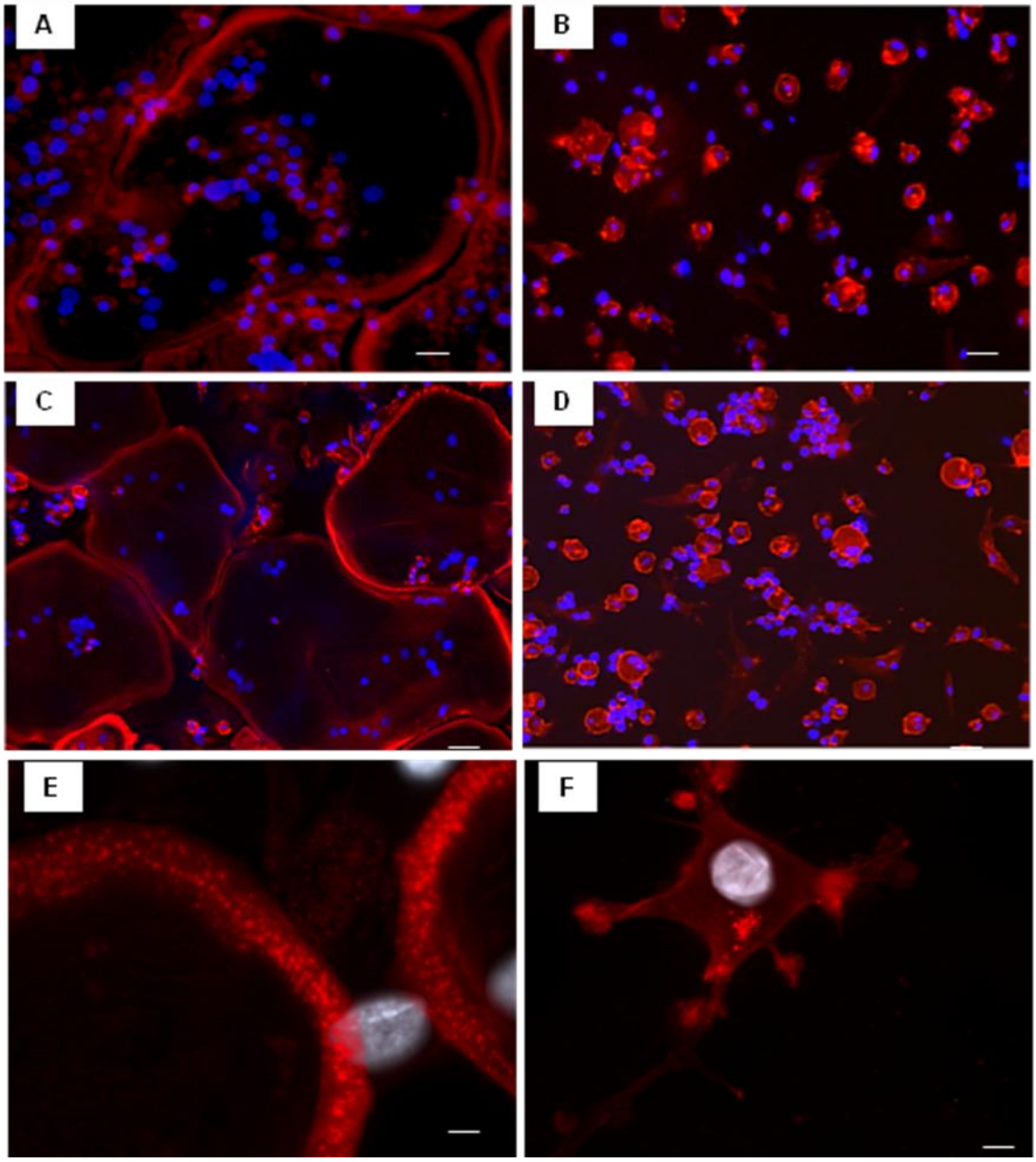
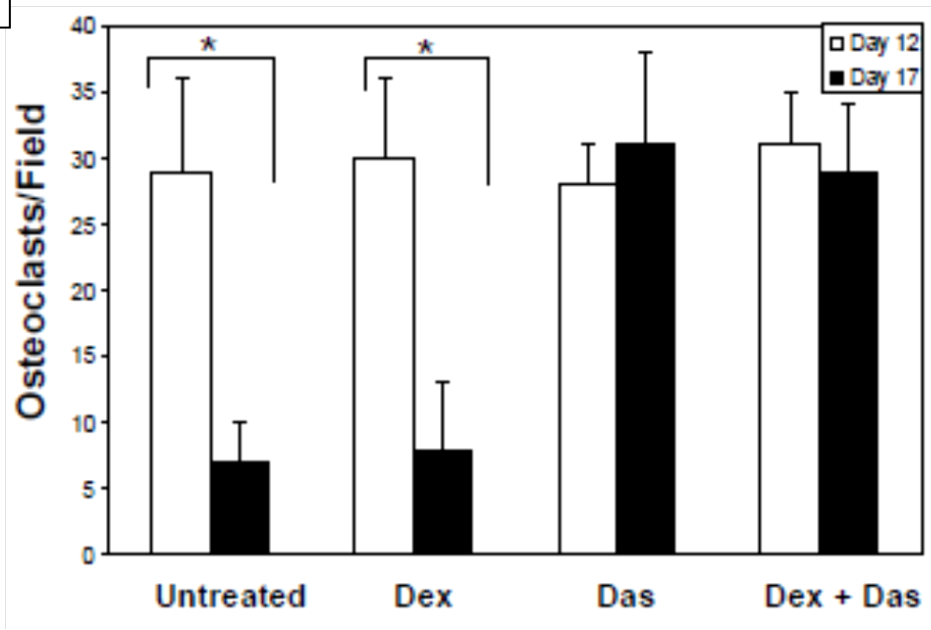

A
B
C
D
E
F

Fig 2: Dexamethasone does not affect osteoclast integrity and function whereas Dasatinib blocks osteoclastogenesis. Osteoclasts were seeded on glass cover slips and at the time of interest they were fixed with 1% PFA, permeabilised with 0.05% Triton X-100 and stained with Alexa568-Phalloidin to detect the distribution of actin filaments (red) and DAPI to detect nuclei (blue). **(A)** Histograms show the average of number of osteoclasts per field using a 10X lens magnification at day 12 and day 17 of differentiation in vitro. As osteoclasts differentiate they fuse and increase in size so that the number of osteoclasts per field decreases. Cultures were left untreated or were treated with 1 uM Dexamethasone (Dex), 150 nM Dasatinib (Das) or the combination of these 2 drugs. **(B)** Histograms showing the average of the number of nuclei per osteoclast at day 12 and day 17 of culture. As osteoclasts differentiate they fuse and the number of nuclei per cell increases. * $p<0.05$ Student t-test

A


Osteoclasts/Field
40
35
30
25
20
15
10
5
0
*
*
Day 12
Day 17
Untreated
Dex
Das
Dex + Das

B

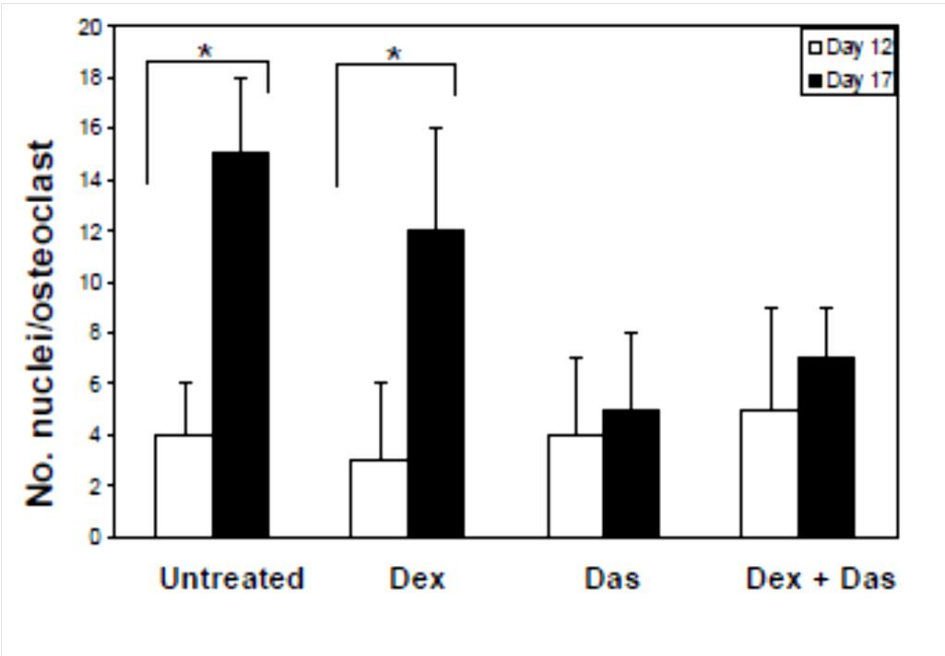

No. nuclei/osteoclast
20
18
16
14
12
10
8
6
4
2
0
*
*
Day 12
Day 17
Untreated
Dex
Das
Dex + Das

Figure 3: Effects of Dasatinib on osteoclasts is reversible. Primary osteoclasts were left Untreated (A) or treated with 150nM Dasatinib (B), 1uM Dexamethasone (C), combination of 150nM Dasatinib and 1uM Dexamethasone (D) for 3 days . Media with drugs were removed after Day 3 and plated in fresh media for another 72 hours (3 days post treatment), showing recovery of osteoclasts in dasatinib and dasatinib and dexamethasone combination wells.

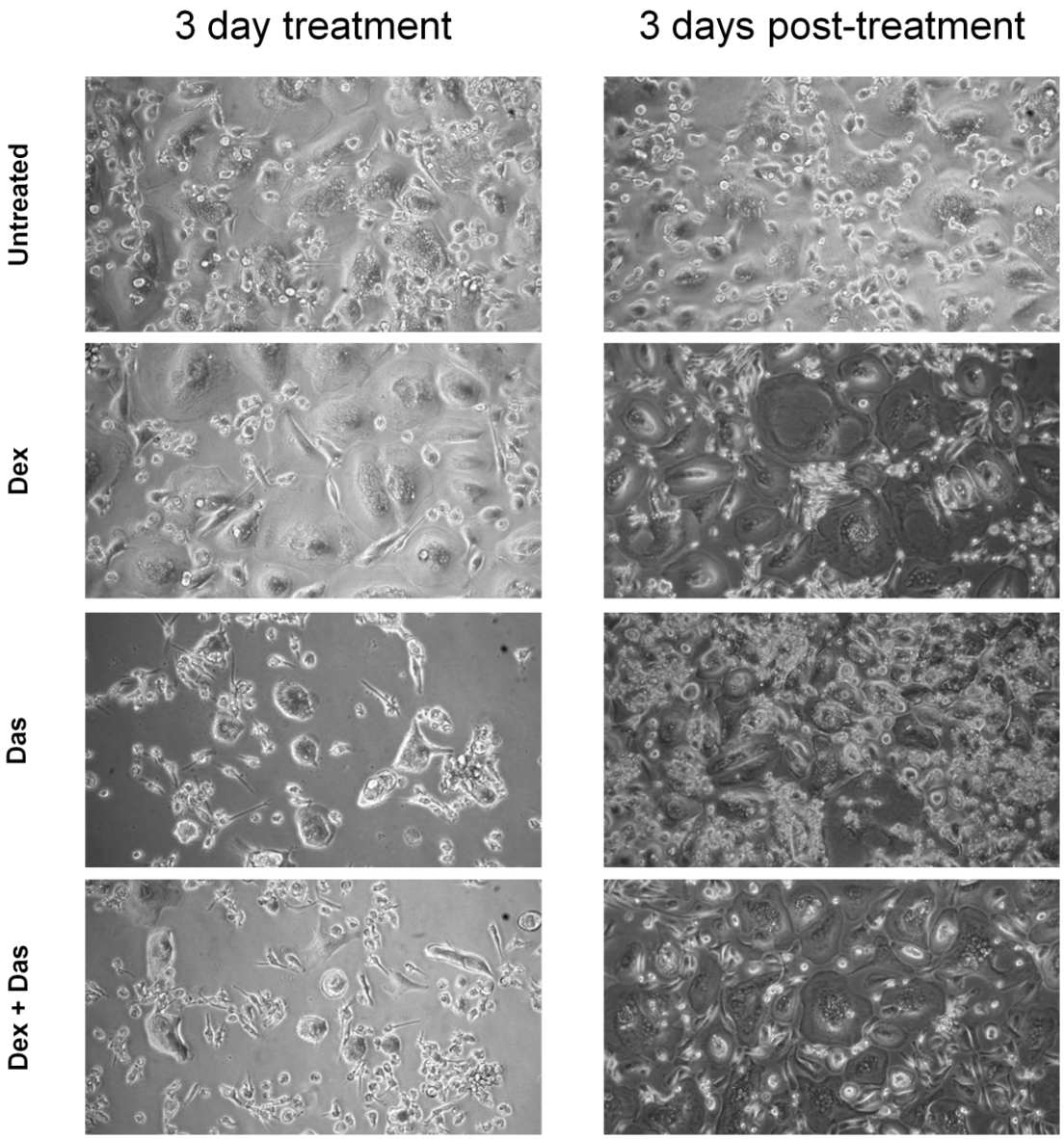
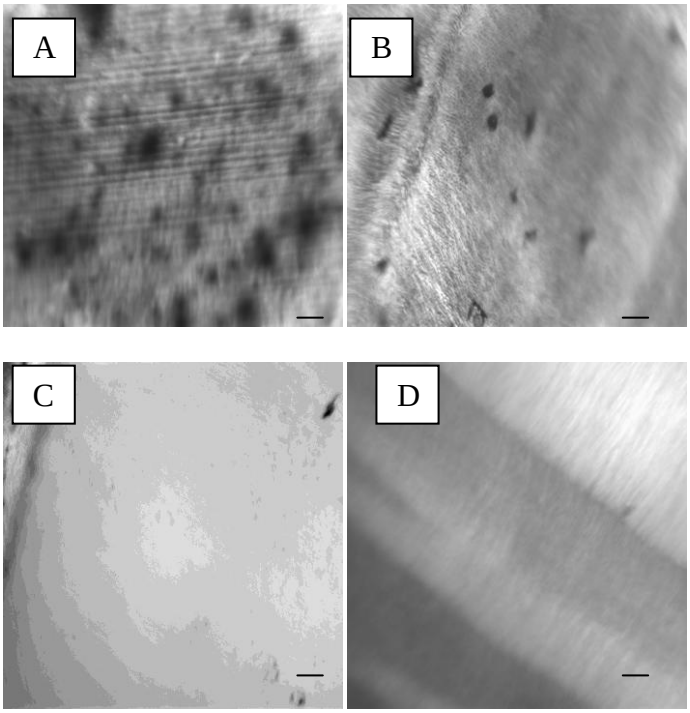
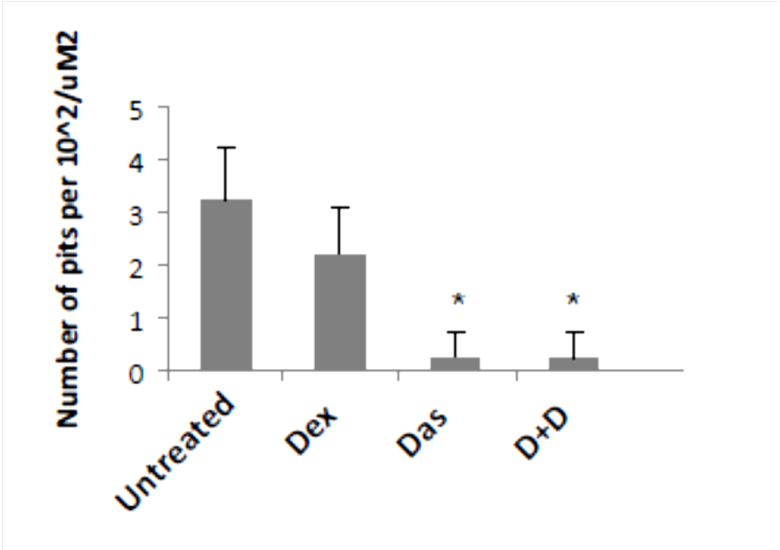

3 day treatment
3 days post-treatment
Untreated
Dex
Das
Dex + Das

Fig 4: Dasatinib inhibits the resorptive function of osteoclasts. Osteoclasts were cultured on dentine discs for 17 days. Cultures were left untreated (A) or were treated with 1 uM Dexamethasone (Dex) (B) , 150 nM Dasatinib (Das) (C) or the combination of these 2 drugs (D) . At day 17 osteoclasts were lysed and dentine discs were sonicated to remove cell debris. The discs were stained with Toluidine to visualise the pits of resorption generated by osteoclasts. Bars (A -D) 20um. The number of pits were analysed by phase contrast microscopy and Histograms show the average number of pits per um^2 in four discs. * $p<0.05$ Student t-test



E



7.4 Effect on plasma cells in coculture with osteoclasts

Yaccoby et al showed that the protection from cell death mediated by osteoclasts on plasma cells is cell – cell contact dependent. In chapter 4 of this thesis, I showed the combination of Dasatinib and dexamethasone was effective in inhibiting proliferation of both Human and murine myeloma cell lines to varying degree. I have also shown in chapter 5 that osteoclasts can protect myeloma cells from dexamethasone induced apoptosis. In this chapter I will test whether Dasatinib may overcome such protection of MM cells against dexamethasone by osteoclasts. In the novel coculture system eGFP expressing plasma cells and osteoclasts were left either untreated or treated with drugs. Both plasma cell proliferation by measuring the fluorescence intensity and the expression of Annexin V as a marker of apoptosis were analysed. MM1S cells which are sensitive to steroids were co cultured with osteoclasts in the presence or absence of drugs. Untreated eGFP labelled MM1S cells either alone or in the presence of osteoclasts proliferated well as evidenced by increased fluorescence intensity and small percentage of cells expressing Annexin V on the cell surface (Fig 5 Panel A, B). The presence of osteoclasts in coculture did not enhance further proliferation in this short term culture (Fig 5 Panel A, B). Treatment with Dasatinib of eGFP MM1S cells alone or in cocultures with osteoclasts did not inhibit proliferation of plasma cells and the level of proliferation and cell death was comparable to untreated cultures (Fig 5 Panel A, B). As expected treatment with dexamethasone inhibited proliferation of plasma cells left alone by inducing cell death through apoptosis observed by higher expression of Annexin V. The presence of osteoclasts in coculture did not enhance or sustain plasma cell proliferation in

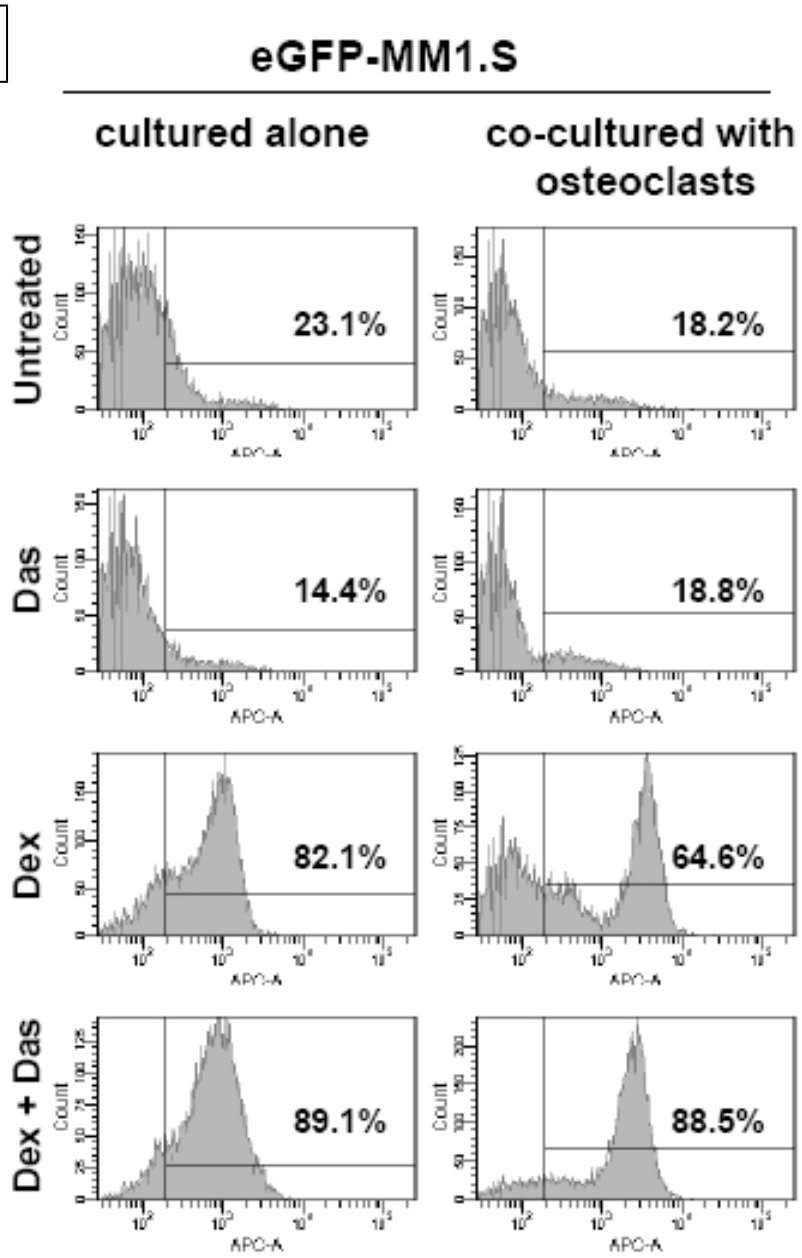
Dex treated wells but partially protected plasma cells from apoptosis (64.6% of apoptotic plasma cells in cocultures with OC's vs. 82.1% of apoptotic cells in cell treated alone) (Fig 5 Panel A, B). The combination of dasatinib and dexamethasone was able to inhibit proliferation as well as induce apoptosis of plasma cells to similar extent in both, plasma cells cultured alone or in coculture with osteoclasts. The drug combination induced 88% of apoptotic fraction of plasma cells when they were in coculture with osteoclasts (Fig 5 Panel A, B). This confirms that the drug combination is able to overcome the protective effect of the osteoclasts observed in dexamethasone alone treatment wells.

MM1R cells are resistant to dexamethasone treatment due to presence of truncated glucocorticoid receptor in these cells. The eGFP labelled MM1R cells left untreated either alone or in the presence of osteoclasts proliferated well as evidenced by increased fluorescence intensity and small percentage of cells expressing Annexin V on the cell surface (Fig .6 Panel A, B). The presence of osteoclasts in coculture did not enhance further proliferation of MM1R cells in the short term cultures. Treatment with Dasatinib of eGFP MM1R cells alone or in cocultures with osteoclasts did not inhibit proliferation of plasma cells. Treatment with Dexamethasone resulted in a minor increase in apoptosis of eGFP-MM1.R cells grown alone (Figure 6 A). However, this reduced viability failed to have an impact on eGFP-MM1.R cell proliferation. Although the apoptotic fraction of plasma cells cultured alone increased with Dasatinib and dexamethasone treatment, cell cycle analysis previously described in Chapter 4 showed an intact proliferative fraction (Fig 6 Panel A, B). Plasma cells in coculture with osteoclasts and treated with dasatinib and

dexamethasone continued to proliferate and remained viable as shown by no increase in Annexin V expressing cells (Fig 6 Panel A, B). Combination of dasatinib and dexamethasone did not significantly affect the proliferation of MM1R plasma cells in the presence of osteoclasts (Fig 6 Panel A, B).

Figure 5. Viability and cell proliferation of MM1S cells on osteoclasts in response to treatment with Dexamethasone and Dasatinib. Histograms showing the levels of staining of Annexin V as detected by flow cytometry in eGFP-MM1.S **(A)** cultured alone or in co-culture with osteoclasts. To generate the histograms of the corresponding cells in co-culture, we acquired 10,000 events in the gated eGFP positive subpopulation. Cultures were left untreated (UT) or were treated with 1 uM Dexamethasone (Dex), 150 nM Dasatinib (Das) or the combination of these 2 drugs for 72 h. Using a fluorescent plate reader, proliferation of eGFP-MM1.S cells **(B)** cultured alone or in the presence of osteoclasts was estimated by measuring the average of fluorescent intensity per well at day 0 and day 4 after plating.

A


eGFP-MM1.S
cultured alone
co-cultured with osteoclasts
Untreated
Das
Dex
Dex + Das
23.1%
18.2%
14.4%
18.8%
82.1%
64.6%
89.1%
88.5%
Count
APC-A

B

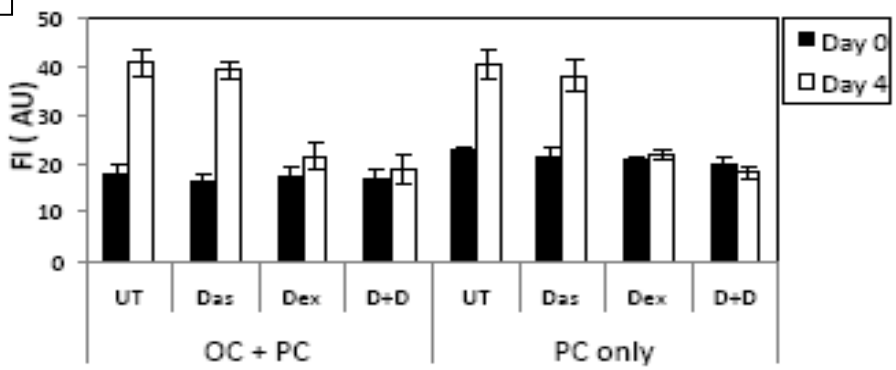
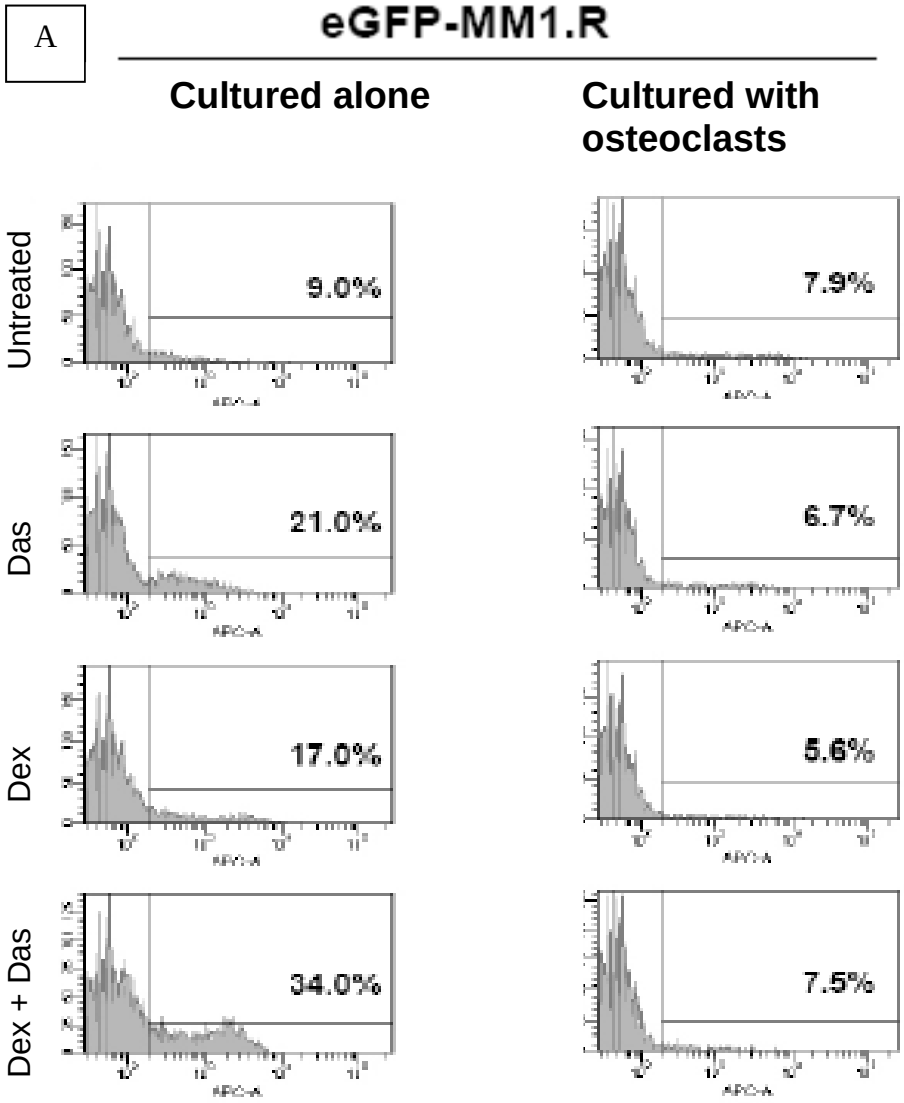
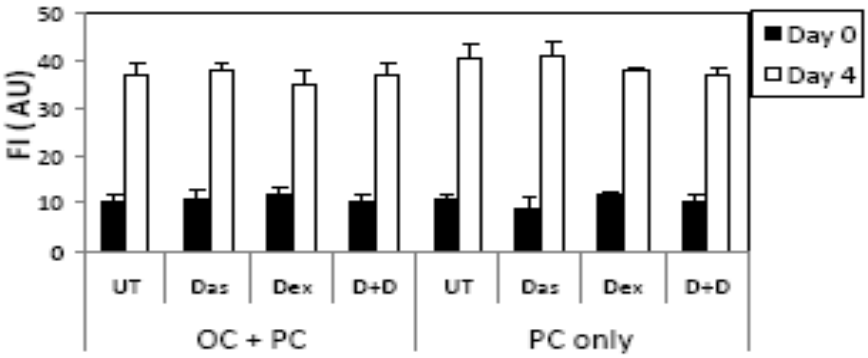

FI (AU)
50
40
30
20
10
0
Day 0
Day 4
UT
Das
Dex
D+D
UT
Das
Dex
D+D
OC + PC
PC only

Figure 6. Viability and cell proliferation of MM1R cells on osteoclasts in response to treatment with Dexamethasone and Dasatinib. Histograms showing the levels of staining of Annexin V as detected by flow cytometry in eGFP-MM1.R **(A)** cultured alone or in co-culture with osteoclasts. To generate the histograms of the corresponding cells in co-culture, we acquired 10,000 events in the gated eGFP positive subpopulation. Cultures were left untreated (UT) or were treated with 1 uM Dexamethasone (Dex), 150 nM Dasatinib (Das) or the combination of these 2 drugs for 72 h. Using a fluorescent plate reader, proliferation of eGFP-MM1.R cells **(B)** cultured alone or in the presence of osteoclasts was estimated by measuring the average of fluorescent intensity per well at day 0 and day 4 after plating.

A


eGFP-MM1.R
Cultured alone
Cultured with osteoclasts
Untreated
Das
Dex
Dex + Das
9.0%
7.9%
21.0%
6.7%
17.0%
5.6%
34.0%
7.5%

B


FI (AU)
50
40
30
20
10
0
Day 0
Day 4
UT
Das
Dex
D+D
UT
Das
Dex
D+D
OC + PC
PC only

7.5 Effect on plasma cells in coculture with stromal cells

Bone marrow stroma protects plasma cell from apoptosis by providing a cellular surface for adhesion and secreting soluble cytokines such as IL-6. Bone marrow stromal cells also induce proliferation of plasma cells. Osteoclasts protected plasma cells from apoptotic cell death when treated with dexamethasone. We wanted to study in the novel *in vitro* coculture system of plasma cells and stromal cells, whether the drugs dexamethasone, dasatinib and the combination have proapoptotic effects. Stromal cells HS5 is a human fibroblastic stromal cell line which is commonly used in cocultures with myeloma cells as it offers protection against therapeutic agents [181]. The eGFP MM1S cells were plated on stromal cells and fluorescence intensity and Annexin V expression of was studied after 72 hours, with or without drug treatment. In the untreated cells there was no enhanced proliferation in the presence of stroma at the cell density described in the methods section. (Fig 7 B). In Dexamethasone treated cultures, the percentage of apoptotic eGFP MM1S cells decreased in the presence of stromal cells, confirming protection (Fig 7A). In the presence of dexamethasone, proliferation of eGFP MM1S cells plated alone fell significantly, but plasma cells in coculture with stromal cells continued to proliferate (Fig 7 B). This was due to the antiapoptotic effect and protective effect of stromal cells in coculture with eGFP MM1S cells (Fig 7 A). Treatment with Dasatinib did not significantly alter cell proliferation or induce apoptosis of eGFP MM1S cells either in the presence or absence of stromal cells. The combination of Dasatinib and dexamethasone induced significant inhibition of proliferation of eGFP MM1S cell line either in the presence or absence of HS5 stromal cells.

This was also evidenced by almost 100% apoptotic eGFP MM1S cells by 72 hours of combined treatment with Dasatinib and dexamethasone. Combination of Dasatinib with Dexamethasone enhanced the percentage of apoptotic eGFP-MM1.S cells in the presence of stromal cells by 3.5-fold in comparison with co-cultures treated with Dexamethasone alone (Fig 7 A).

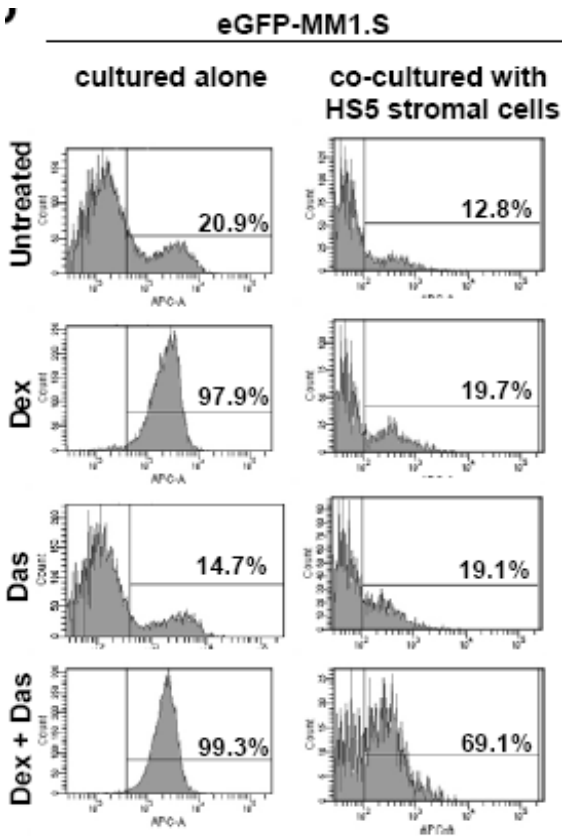
The eGFP MM1R cells are resistant to Dasatinib and dexamethasone at the current concentrations described which are clinically achievable. eGFP-MM1R plasma cells continued to proliferate in the presence of drugs (Fig7 Panel B). Flow cytometric analysis confirmed the findings on the proliferation assay with no increase in apoptotic eGFP MM1R cells cultures alone or in cocultures with either dasatinib, dexamethasone (Fig 8 A). The combination of dasatinib and dexamethasone had no significant effect on proliferation of eGFP MM1R cells. But expression of Annexin V was modestly increased when myeloma cells were grown alone with some reduction of apoptotic eGFP MM1R cells in coculture with HS5 stromal cells (Fig 8 A). Dexamethasone enhanced the proliferation of mCherry-HS5 cells in co-culture with either MM1S or MM1R cells. 150 nM Dasatinib significantly reversed dexamethasone-enhanced proliferation of mCherry-HS5 cells co-cultured with MM cells (Figure 7 C). The effect of drugs on the stromal cells was also studied by flow cytometry alone and in coculture with stromal cells. The eGFP expression of plasma cells were used to gate out the myeloma cells in coculture. Treatment with Dasatinib, dexamethasone or the combination had no significant proapoptotic effect in the stromal cells at the drug concentrations used for up to 72 hours (Fig 8 B). Similar effect was observed when stromal cells were co cultured with eGFP MM1S cells and treated with the same drug treatments (Fig 8 Panel B).This

confirms that these drugs are unlikely to induce stromal cell death in vivo which may be important when therapeutic potential of drugs are considered.

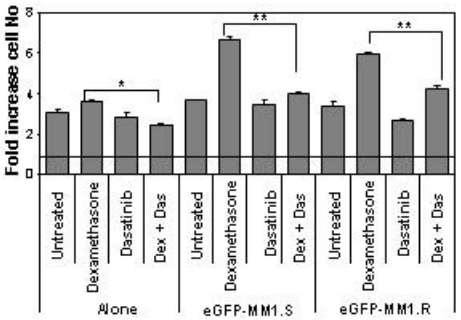
Figure 7. Sensitisation of eGFP-MM1.S cells to Dexamethasone in the presence of stromal cells by combined treatment with Dasatinib.

(A) Histograms showing the levels of staining of Annexin V as detected by flow cytometry in eGFP-MM1.S cells alone or in co-culture with HS.5 cells. The eGFP-MM1.S myeloma cells and the cocultures were left untreated or treated with 1 uM Dexamethasone (Dex), 150 nM Dasatinib (Das) or the combination of these 2 drugs To generate the histograms of the corresponding cells in co-culture, 10,000 events in the gated subpopulation of interest was acquired. **(B)** Histograms showing the fold increase in proliferation of mCherry HS 5 stromal cells cultured alone in the presence and absence of drugs and in co culture with eGFP-MM1.S and eGFP-MM1.R plasma cells. (**C**) Histograms showing the fold increase in proliferation of eGFP-MM1.S and eGFP-MM1.R cells in co-culture with stromal cells in the presence and absence of drugs * $p<0.05$; **$p<0.01$; $p<0.005$, Student test.

A


eGFP-MM1.S
cultured alone
co-cultured with HS5 stromal cells
Untreated
Dex
Das
Dex + Das
20.9%
12.8%
97.9%
19.7%
14.7%
19.1%
99.3%
69.1%

B


Fold increase cell No
Untreated
Dexamethasone
Dasatinib
Dex + Das
Alone
eGFP-MM1.S
eGFP-MM1.R

C

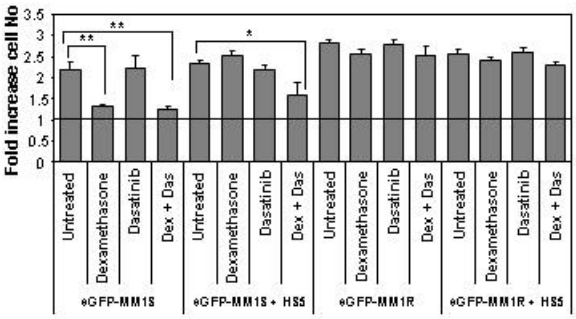
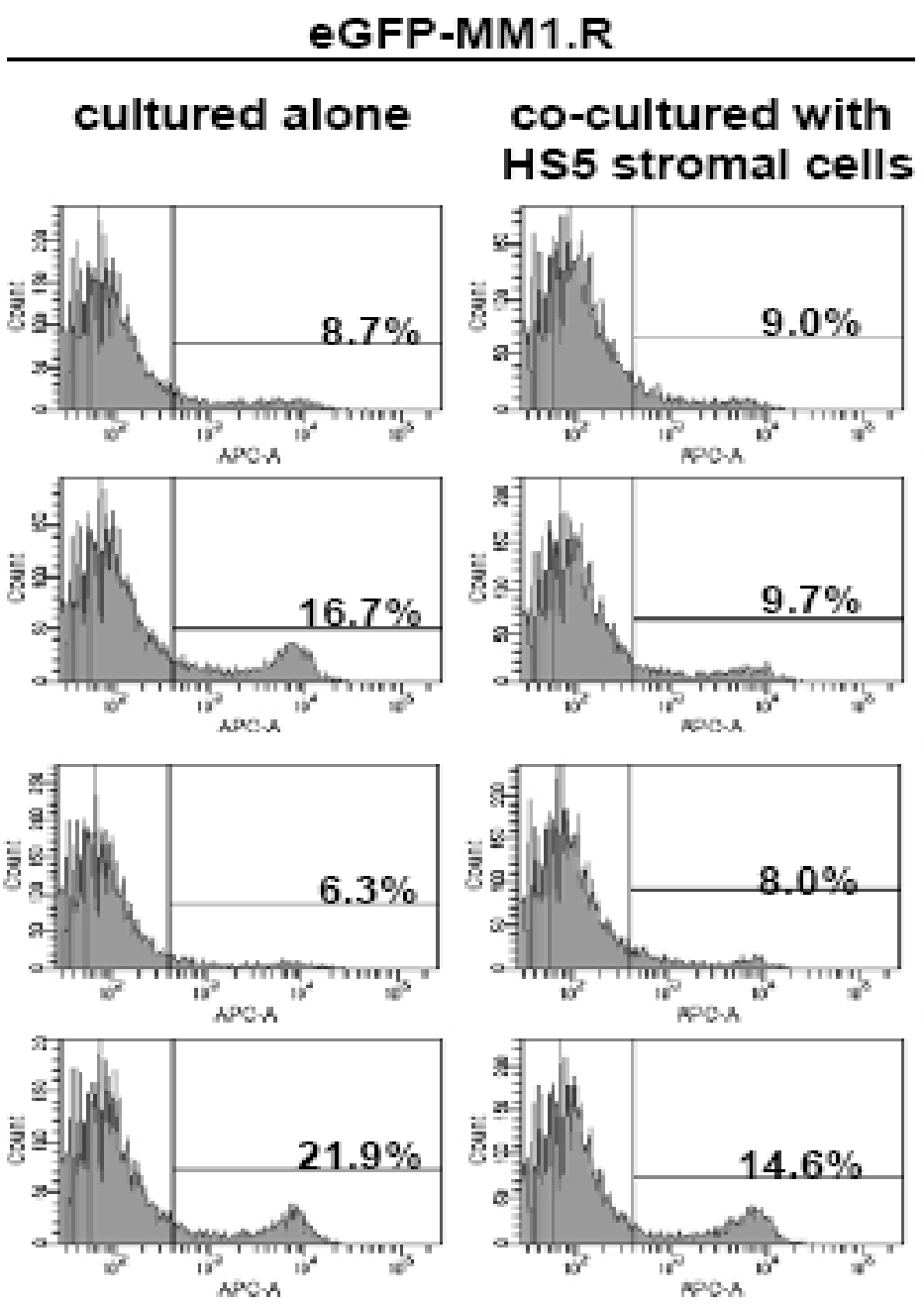

Fold increase cell No
Untreated
Dexamethasone
Dasatinib
Dex + Das
eGFP-MM1S
eGFP-MM1S + HS5
eGFP-MM1R
eGFP-MM1R + HS5

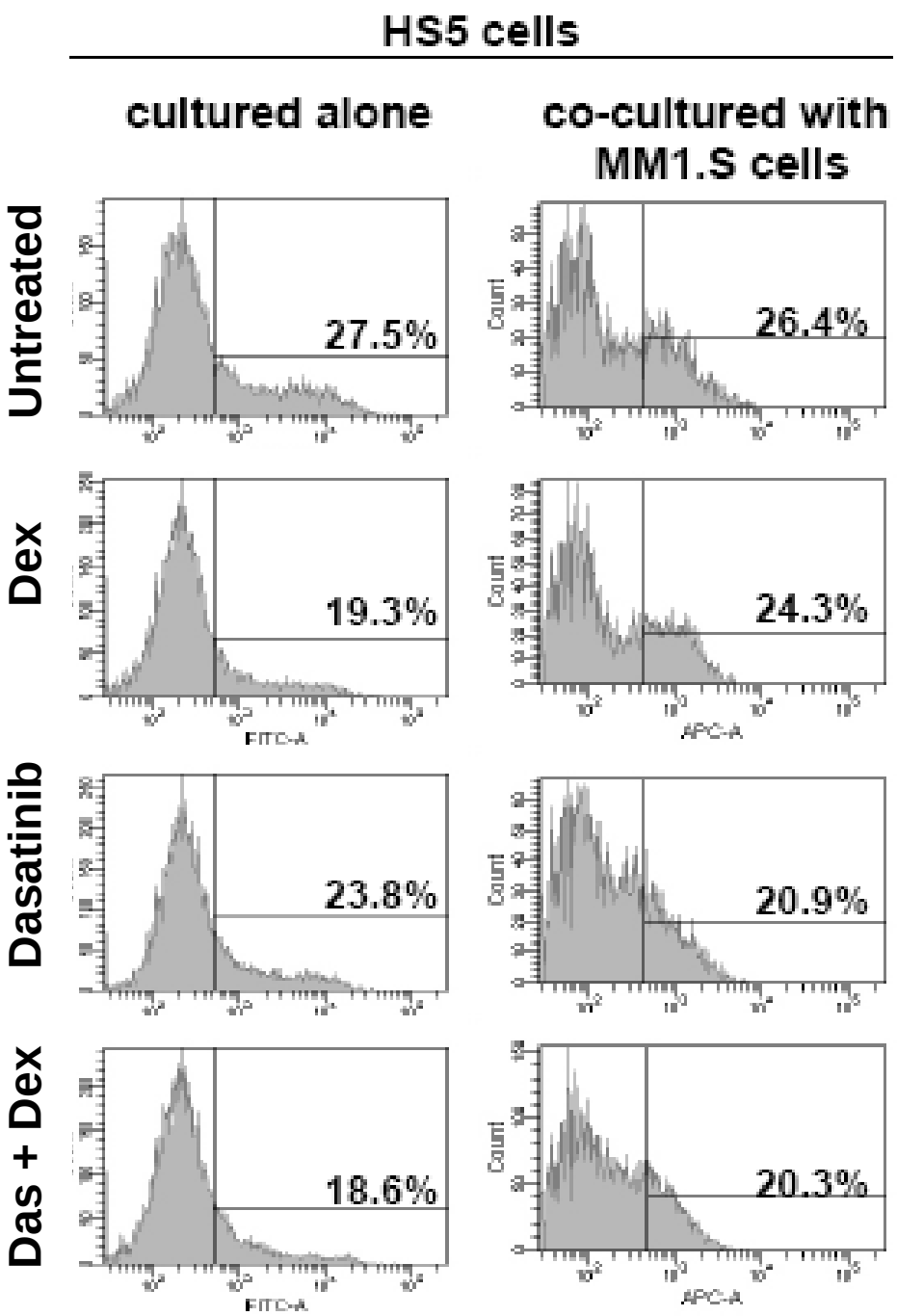
Figure 8: Effect of drugs on HS5 stromal cells and steroid resistant eGFP MM1R cells cultured alone or in coculture with stromal cells

(A) Histograms showing the levels of staining of Annexin V as detected by flow cytometry in eGFP-MM1.R cells alone or in co-culture with HS.5 cells. The eGFP-MM1.Rmyeloma cells and the cocultures were left untreated or treated with 1 uM Dexamethasone (Dex), 150 nM Dasatinib (Das) or the combination of these 2 drugs To generate the histograms of the corresponding cells in co-culture, 10,000 events in the gated subpopulation of interest was acquired. **(B)** Histograms showing the levels of staining of Annexin V as detected by flow cytometry in HS5 cells alone or in co-culture with eGFP MM1S cells. To generate the histograms of the corresponding cells in co-culture, up to 10,000 events were acquired in the gated subpopulation of interest (eGFP positive for MM cells and eGFP negative for HS5 cells).

A


eGFP-MM1.R
cultured alone
co-cultured with HS5 stromal cells
8.7%
9.0%
16.7%
9.7%
6.3%
8.0%
21.9%
14.6%
Count
APC-A

B


HS5 cells
cultured alone
co-cultured with MM1.S cells
Untreated
Dex
Dasatinib
Das + Dex
27.5%
26.4%
19.3%
24.3%
23.8%
20.9%
18.6%
20.3%
Count
FITC-A
APC-A

7.6 Discussion:

I have shown in this chapter that Dasatinib at clinically achievable concentrations significantly inhibits osteoclastogenesis. Presence of dasatinib also inhibits osteoclast function. My data show that dasatinib causes major disruption of the osteoclast cytoskeleton, which correlates with inhibition of bore resorption and cell fusion. The correlation between abnormal organisation of the osteoclast cytoskeleton and impairment of osteoclast cell fusion and bone resorption is expected as they require an intact organisation and adequate dynamics of F-actin. Dasatinib is a potent c-Src inhibitor and at 1nM concentrations it also inhibits c-fms and its downstream signalling significantly in the presence of M-CSF, a key cytokine involved in osteoclastogenesis [214]. The crucial role of c-Src in osteoclast function through the organisation of the actin cytoskeleton has been confirmed in vitro and in vivo[215,216]. There is also evidence suggesting the c-Abl could be involved in osteoclast function. For example, Imatinib has been shown to block osteoclastogenesis[138]. Imatinib promotes osteoblast differentiation by inhibiting PDGFR signalling and inhibits osteoclastogenesis by both direct and stromal cell-dependent mechanisms and long term Imatinib therapy administered in chronic myeloid leukaemia patients showed evidence of impairment in bone remodelling[139]. In vitro data indicate that dasatinib block osteoclast function and this is further supported by several in vivo studies. Dasatinib treated sprague-dawley rats exhibited evidence of osteoclast inhibition which was comparable to rats treated with zoledronic acid. No significant increase in osteoblastic activity was observed[217]. Dasatinib has also been previously shown to inhibit osteoclast activity in vitro and in vivo and

in the presence of prostate cancer cells[217,218]. Long term dexamethasone administration in patients causes osteoporosis. In my experiments, Dexamethasone did not affect osteoclastogenesis or osteoclast function. Dexamethasone promotes osteoclastogenesis with help of marrow adipocytes and also works synergistically with TGFβ which has been shown to be increased in sera of myeloma patients[219,220]. These results suggest that treatment of MM patients with dexamethasone would not resolve bone disease. Our data may imply that through the inhibition of osteoclast function, dasatinib would be a beneficial drug to target bone disease while promoting the pro-apoptotic efficacy of dexamethasone. Autologous and allogeneic osteoclasts have been shown to protect plasma cell from apoptosis through cell-cell contact [49].In the above experiments although osteoclast protected plasma cells from apoptosis on exposure to dexamethasone, the combination of dasatinib and dexamethasone overcame this protection. This effect could partly be mediated through reduced plasma cell adhesion to osteoclasts and also reduction in available osteoclast surface for plasma cells to interact, due to the effect of dasatinib on the osteoclast size. Although Dasatinib affects osteoclastogenesis, removal of drug and regrowing cells in presence fresh media was able to recover osteoclast formation and function. This is critical to maintain the bone homeostasis in myeloma patients, particularly because of the chronic nature of the disease. Due to the role of osteoclasts in haematopoiesis, recovery of osteoclast function could facilitate the repopulation of the BM after drug treatment. Bone marrow stroma protection of myeloma plasma cells in the presence of drugs has been frequently demonstrated in vitro. We were able to reliably demonstrate the effects on

plasma cells in the presence of BM cells (HS5 BM fibroblastic cells and osteoclasts) with the help of the validated eGFP labelled plasma cell coculture system. In my experiments HS5 stromal cells protected myeloma cell lines from pro apoptotic effects of dexamethasone. But combination of dasatinib and dexamethasone was able to overcome these effects. This effect of dasatinib is not mediated through inhibition of stromal cell viability, but by inhibition of adhesion and secretion of IL-6. Both Dasatinib and dexamethasone or the combination does not induce apoptosis in HS5 stromal cells during short term cultures. This has clinical relevance as bone marrow stroma is required to support haematopoiesis to prevent toxicity in patients receiving therapy. I also found that Dexamethasone treatment enhanced the proliferation of mCherry-HS5 cells in the presence of eGFP-MM cells. This could be reversed by co-treatment of dexamethasone with dasatinib and correlated with sensitisation of MM cells to dexamethasone-induced apoptosis. This can be explained by the dependence of proliferation of stromal cells on Src and c-Abl kinases [221,222].

In this chapter, the effects of Dasatinib on both osteoclasts and SCs have been demonstrated. Dexamethasone promotes osteoclastogenesis and stromal cells are able to protect myeloma cells from apoptosis. Dasatinib overcomes these effects and in combination induces significant apoptosis in myeloma cell lines. Also by targeting adhesion of MM cells to SCs by Dasatinib and dexamethasone combination could decrease bone disease by interfering with the physical contact required to induce secretion of DKK1 and RANKL by SC leading to osteoclast activation. The effects of soluble and cellular components of the microenvironment on myeloma tumour cells is

diverse [54]. Drugs which have specific targets in the microenvironment are unlikely to have significant impact because the microenvironment will support early tumour regrowth. Multitargeted agents such as Dasatinib, with effects on plasma cell adhesion, osteoclastogenesis, overcoming stromal protection and sensitising myeloma plasma cells to dexamethasone, may offer hope to patients.

DISCUSSION AND

FUTURE WORK

8.0 Discussion and Future work

MM remains an incurable disease with a relapsing remitting course until disease becomes resistant to all therapies. Intramedullary myeloma growth is closely linked to development of bone damage which is a key clinical feature leading to morbidity in this disease. In a majority of patients relapses occur with further evidence of new bone damage. This also partly explains the improved survival observed in the myeloma IX trial in the cohort of patients randomised to receive Zoledronic acid an amino bisphosphonate which significantly inhibits osteoclastic activity.

CAM-DR promotes resistance in myeloma and to study these processes better, in vitro models are required to recreate the in vivo setting. I have developed a new experimental model combined with the work of other people in the group (mCherry-HS5 cells). Using this model I demonstrated that fibroblastic stromal cells and OC protect MM cells against Dex and this can be overcome with Dasatinib. The data confirmed the importance of analysing both plasma cell proliferation and viability (apoptosis/necrosis) to fully determine the effect of drugs in coculture experiments. The data showed the protection against Dex when plasma cells were grown in coculture with OCs. In the presence of OCs, Dex still blocks MM cells proliferation but I was still able to detect a small viable population of cells which is not present when the cells are treated alone. It is quite likely that this viable population could emerge as a resistant clone and would continue to proliferate after drug withdrawal.

My data indicates sensitisation of MM cells to Dexamethasone by Dasatinib in the presence of HS5 stromal cells was achieved through: a) inhibition of MM

cell adhesion; b) inhibition of IL-6 secretion; c) synergy of the effect of Das and Dex to induce apoptosis in MM cells. This could be attributed to the role of Src family kinases in transduction of Dex-signalling. Inhibition of Lck enhances glucocorticoid sensitivity and apoptosis in lymphoid cell lines and in chronic lymphocytic leukemia[223], d) Inhibition of Dexamethasone-induced fibroblastic stromal cell proliferation. Dexamethasone has been shown to induce stromal proliferation in prostatic and adipose tissues both in vitro and in vivo[221,224]. Activation of Src signalling underlies dexamethasone induced stromal cell proliferation. In the treatment of myeloma dexamethasone has been shown to be a useful adjunct in increasing responses to both IMiDs and Proteasomal inhibitors. Although direct anti myeloma effects are more pronounced by the addition of dexamethasone, potential niches of resistance can be simultaneously induced.

Addition of Dasatinib resulted in sensitisation of MM cells to Dexamethasone in the presence of OCs through inhibition of adhesion of MM cells. Dasatinib therapy could be effective in blocking MM bone disease as it inhibits bone resorption activity of mature OCs through disruption of the actin cytoskeleton. These data indicate that targeting the OC cytoskeleton is a possible strategy to block bone disease in MM. Mouse Knockouts of proteins involved in the organisation of the OC cytoskeleton such as Src itself, WASP and gelsolin results in alteration of osteoclast function *in vivo*[114,225]. Dasatinib also by inhibiting c-Src expression aids differentiation of mesenchymal stromal cells into osteoblasts resulting in bone formation[226,227]. Bortezomib is the only currently available agent which has shown this effect in myeloma patients in vivo[52].

I have also demonstrated that stromal cells have the capacity to induce proliferation of myeloma cells in coculture, which is not observed when myeloma cells are coculture with osteoclasts. Osteoclasts both autologous and allogeneic have been previously shown to enhance myeloma survival in cocultures [49]. The observation of increased proliferation of myeloma cells in stromal cocultures could be due to presence of higher levels of IL-6 in these cocultures.

This data suggest the combination of Dasatinib and dexamethasone would be synergistic in enhancing responses in myeloma patients. More recently a similar approach has been taken with use of plerixafor, a CXCR4 inhibitor which overcomes the protection from the stromal environment [95]. A Phase I trial has been initiated with the combination of bortezomib and plerixafor in relapsed refractory myeloma patients.

Myeloma patients continue to relapse despite previous response to therapy. It is currently thought that both intrinsic genetic changes in plasma cells and microenvironmental changes account for this. The potential interest in exploring this combination in myeloma patients is the little or no direct antimyeloma activity of Dasatinib in this combination[136]. This would be one of the first combinations with an anti-microenvironmental agent to be trialled in myeloma patients apart from plerixafor. As all MM patients would have previously been exposed to dexamethasone, it would be easier to observe synergistic in vivo combination of these two agents The prominent in vitro effect on osteoclasts is particularly advantageous in this combination as dexamethasone induces osteoporosis. As there are established frontline combinations in the treatment of myeloma inducing responses in the order of

70 -80%, the role of this combination will be limited or more relevant in the relapsed refractory population, particularly in patients with active bone disease. Relapsed/ Refractory myeloma patients are heavily pre-treated and suffer from considerable comorbidities because of age and the disease per se. Although this is an oral combination they are not without significant side effects as shown in other trials. Higher doses of dexamethasone which will be relied upon as the anti MM agent has been shown to have adverse overall survival in this group of patients[228]. Dasatinib used in chronic myeloid leukaemia has also demonstrated manageable but some significant side effects in this group of patients [229]. A phase I/ II dose escalation study of Dasatinib with dexamethasone with escalating cohorts of Dasatinib would be the best design to explore this combination further. Based on the *in vitro* data patients with skeletal morbidity or with multiple skeletal plasmacytomas may benefit from this combination. This trial could also explore if drugs with strong anti micro environmental effect were added to treatment combination results in lowering of dexamethasone doses.

Future work:

1) Explore further combinations with dasatinib on the high throughput platform
2) Little is known about the changes in the stromal cell compartment of myeloma patients. The in vitro model could be used to study the changes in the stromal cell compartment and osteoclasts, with drugs in myeloma patient samples.
3) Intracellular pathways activated in adherent plasma cells on fibronectin and stromal cells. Using GEP and phosphoproteomics other key mediators in cell signalling pathways downstream of the integrin receptors could be identified and targeted.
4) Dasatinib has been shown to reduce tumour load in SCID mouse models injected with myeloma cell lines. Profound changes in osteoclasts in presence of dasatinib were observed in vitro and reported in bone biopsies of CML patients on long term dasatinib. The in vivo effects on bone disease could be evaluated in C57/Bl KawlriJ syngeneic mouse myeloma model which develops bone disease within 4 weeks after tumour injection[160].
5) Dasatinib is a standard therapy in chronic myeloid leukaemia patients. This would make it easier to develop a Phase I/II study to look at safety, tolerability of combination of dasatinib and dexamethasone in relapsed/ refractory myeloma patients. Tumour responses, toxicity and bone changes by PET activity and biochemistry would be the evaluable outcomes in this study.

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